REC'D **0 6 JUL 2004**

| WIPO | PCT |

**Patent Office
Canberra**

I, LEANNE MYNOTT, MANAGER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2003902875 for a patent by THE UNIVERSITY OF MELBOURNE as filed on 10 June 2003.

WITNESS my hand this
Twenty-third day of June 2004

LEANNE MYNOTT
MANAGER EXAMINATION SUPPORT
AND SALES

**PRIORITY
DOCUMENT**
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

- 1 -

IMMUNOMODULATING COMPOSITIONS, USES THEREFOR AND PROCESSES FOR THEIR PRODUCTION

FIELD OF THE INVENTION

THIS INVENTION relates generally to modulation of immune responses. More particularly, the present invention relates to the use of at least one set of peptides in compositions and methods for modulating an immune response to one or more polypeptide antigens, wherein the sequences of a respective set of peptides are derived in whole or in part from a single polypeptide antigen. Individual peptides of a respective peptide set comprise different portions of an amino acid sequence corresponding to a single polypeptide antigen and display partial sequence identity or similarity to at least one other peptide of the same set of peptides. The invention also extends to methods of using such peptides in a range of preventive, diagnostic and therapeutic applications.

Bibliographic details of various publications numerically referred to in this specification are collected at the end of the description.

BACKGROUND OF THE INVENTION

Since its discovery almost 20 years ago, the human immunodeficiency virus type-1 (HIV-1) has claimed more than 22 million lives and is continuing to devastate communities worldwide (1). Forty-two million people are currently living with HIV-1 and, despite efforts to modify high-risk behaviour, an estimated 5 million new infections occurs yearly (2). A safe and effective preventative or therapeutic vaccine is desperately needed.

The elimination of persistent intracellular pathogens such as replicating viruses requires the mobilisation of cell-mediated immunity (CMI). CD8+ cytotoxic T lymphocytes (CTL) are the primary effector cells of CMI; they kill virally-infected cells by recognising viral peptides presented on the cell surface in the context of MHC class I molecules. Prior to the appearance of virus-specific antibodies, a robust HIV-1-specific CTL response temporally correlates with reduced viremia during the acute stage of HIV-1 infection (3, 4). Furthermore, strong CTL responses are associated with reduced HIV-1 viremia during chronic infection (5, 6), whereas a decline in HIV-1-specific CTL is linked to rapid progression to AIDS (4, 7-9).

- 2 -

Early HIV-1 vaccine strategies were based on whole inactivated virus and recombinant structural proteins such as the envelope (env) glycoprotein. Non-human primate models revealed only limited strain-specific protection by these vaccines against pathogenic simian immunodeficiency virus (SIV) and highly pathogenic SHIV (SIV-HIV-1 chimeric) challenges (10-13). The first human phase III trials also failed to show efficacy (14).

Particle- and recombinant whole protein-based vaccines, although safe, favour the generation of antibodies that are of insufficient protection. Alternatively, intracellularly expressed antigens are subsequently more likely to induce CTL responses. Live-attenuated viruses generate potent cell-mediated immunity (CMI) responses, however their clinical safety is of concern (15). Consequently, much focus has shifted toward genetically engineered vectors (such as DNA plasmids and poxviruses) expressing HIV-1/SIV genes (such as *env*, *gag* and *pol*) (16).

It is not known which immune-target antigens are protective, but a large breadth of T cell responses has been shown to reduce the opportunity for escape mutants to arise (17). It is this large breadth of potential epitopes, however, which renders the construct of large vectors frequently difficult and as well as potential safety issues. Concerns have been raised about the potential ability of DNA vaccines to integrate with host DNA, as well as the safety of viral vector vaccines in immunocompromised hosts. These represent the significant regulatory hurdles for these recombinant vaccines.

Also, despite significant advances towards understanding how T and linear B cell epitopes are processed and presented to the immune system, the full potential of epitope-based vaccines has not been fully exploited. The main reason for this is the large number of different T cell epitopes, which must be identified for inclusion into such vaccines to cover the extreme human leucocyte antigen (HLA) polymorphism in the human population.

Infusion of whole antigen-pulsed or single epitope-pulsed antigen presenting cells (APC) has previously been reported to be immunogenic in murine models (22-27). However, other reports in inbred mouse models suggest the infusion of cells pulsed with single peptides may even be tolerogenic (induces a state of tolerance to the antigen which would be counterproductive for a vaccine) (28-31).

SUMMARY OF THE INVENTION

The present invention discloses the discovery that autologous cells, which have been contacted with overlapping peptides spanning a viral polypeptide antigen of interest produce a strong immunogenic response in an outbred population that protects against

5   subsequent viral challenge. The present inventors propose that similar protective responses would be achieved using systemic administration of the overlapping peptides *per se*. The use of multiple overlapping peptides provides several advantages, including reducing the emergence of escape mutants and the facile production of peptide-based immunogenic compositions without prior knowledge of any epitopes. In this regard, the sequence overlap

10   between peptides reduces or prevents loss of potential epitopes, which broadens the immunological coverage of the composition to cover potentially the diversity in the major histocompatability complex across an outbred population.

Accordingly, in one aspect of the present invention, there is provided at least one set of peptides for modulating an immune response to one or more polypeptides of interest.

15   Individual peptides of a respective set comprise different portions of an amino acid sequence corresponding to a single polypeptide of interest and display partial sequence identity or similarity to at least one other peptide of the same set of peptides. In certain embodiments, at least 2, 3, 4, 5, 6, 7 sets of peptides are employed, wherein peptide sequences for each set are derived from a distinct polypeptide of interest.

20   The partial sequence identity or similarity is typically contained at one or both ends of an individual peptide. Suitably, at one or both of these ends there are at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 contiguous amino acid residues whose sequence is identical or similar to an amino acid sequence contained within at least one other of the peptides.

In certain embodiments, the peptide is at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20,

25   25, 30 amino acid residues in length and suitably no more than about 500, 200, 100, 80, 60, 50, 40 amino acid residues in length.

In certain embodiments, the peptide sequences are derived from at least about 30, 40, 50, 60, 70, 80 90, 91, 92, 93, 94. 95, 96, 97, 98, 99% of the sequence corresponding to the polypeptide of interest.

30   The polypeptide of interest is suitably an antigen selected from a protein antigen, a particulate antigen, an alloantigen, an autoantigen or an allergen, or an immune complex. In certain embodiments, the polypeptide of interest is a disease- or condition-associated

- 4 -

polypeptide such as but not limited to a polypeptide produced by a pathogenic organism or a cancer. Examples of pathogenic organisms include, but are not restricted to, yeast, viruses, bacteria, helminths, protozoans and mycoplasmas. Examples of cancers include, but are not restricted to, melanoma, lung cancer, breast cancer, cervical cancer, prostate

5   cancer, colon cancer, pancreatic cancer, stomach cancer, bladder cancer, kidney cancer, post transplant lymphoproliferative disease (PTLD), Hodgkin's Lymphoma and the like.

In another aspect, the invention provides antigen-presenting cells or their precursors which have been contacted with a set of peptides as broadly described above for a time and under conditions sufficient for the peptides or processed forms thereof to be

10   presented by the antigen-presenting cells or by their precursors.

In a related aspect, the invention provides a process for producing antigen-presenting cells for modulating an immune response to a polypeptide of interest. The process generally comprises contacting antigen-presenting cells or their precursors with at least one set of peptides as broadly described above for a time and under conditions

15   sufficient for the peptides or processed form thereof to be presented by the antigen-presenting cells or by their precursors. Suitably, when precursors are used, the precursors are cultured for a time and under conditions sufficient to differentiate antigen-presenting cells from the precursors.

In one embodiment, the or each set of peptides is contacted with substantially

20   purified antigen-presenting cells or their precursors. In another embodiment, the or each set of peptides is contacted with a heterogeneous population of antigen-presenting cells or their precursors. In this embodiment, the heterogenous pool of cells can be blood or peripheral blood mononuclear cells. Typically, the antigen-presenting cells or their precursors are selected from monocytes, macrophages, cells of myeloid lineage, B cells,

25   dendritic cells or Langerhans cells.

The antigen-presenting cells broadly described above are also useful for producing lymphocytes, including T lymphocytes and B lymphocytes, for modulating an immune response to a specified antigen or group of antigens. Accordingly, in yet another aspect, the invention provides a method for producing antigen-specific lymphocytes . The

30   method comprises contacting a population of lymphocytes, or their precursors, with an antigen-presenting cell as broadly described above for a time and under conditions

- 5 -

sufficient to produce the antigen-specific lymphocytes that modulate an immune response to at least one polypeptide from which the overlapping peptides were derived.

In yet another aspect, the invention contemplates a composition comprising at least one set of peptides, or the antigen-presenting cells, or the lymphocytes, as broadly described above, and a pharmaceutically acceptable carrier and/or diluent. In certain embodiments, the composition may further comprise an adjuvant.

In yet another aspect, the invention embraces a method for modulating an immune response to a polypeptide of interest, comprising administering to a patient in need of such treatment at least one set of peptides, or the antigen-presenting cells, or the lymphocytes, or the composition as broadly described above for a time and under conditions sufficient to modulate the immune response.

In a related aspect, the invention encompasses a method for treatment and/or prophylaxis of a disease or condition associated with the presence of a polypeptide of interest, comprising administering to a patient in need of such treatment or prophylaxis an effective amount of at least one set of peptides, or the antigen-presenting cells, or the lymphocytes, or the composition as broadly described above. In some embodiments, peptides or antigen-presenting cells or the lymphocytes are administered systemically, typically by injection.

In still yet another aspect, the invention contemplates the use of at least one set of peptides, or of the antigen-presenting cells, or of the lymphocytes, as broadly described above, in the preparation of a medicament for modulating an immune response to a polypeptide of interest or for treating or preventing a disease or condition associated with the presence of a polypeptide of interest.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagrammatic representation showing one embodiment of an SIV$_{mac236}$ gag peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in

5   brackets left of the peptide sequence. Peptide 125 is 14 amino acids in length. The full-length gag sequence is modified from the HIV sequence database _http://hiv-web.lanl.gov_.

Figure 2 is a diagrammatic representation showing one embodiment of an SIV$_{mac236}$ pol peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in

10   brackets left of the peptide sequence. The full-length pol sequence is modified from the HIV sequence database _http://hiv-web.lanl.gov_.

Figure 3 is a diagrammatic representation showing one embodiment of an SIV$_{mac236}$ nef peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in

15   brackets left of the peptide sequence. The full-length nef sequence is modified from the HIV sequence database _http://hiv-web.lanl.gov_.

Figure 4 is a diagrammatic representation showing one embodiment of an SHIV$_{SF162P3}$ env peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically,

20   indicated in brackets left of the peptide sequence. Peptide 211 is 14 amino acids in length. *Peptide overlaps preceding peptide by 10 amino acids to eliminate a forbidden Q n-terminal peptide. The full-length env sequence is modified from the HIV sequence database _http://hiv-web.lanl.gov_.

Figure 5 is a schematic representation of an _in vivo_ CTL killing assay performed

25   at weeks 10, 15 and 20.

Figure 6 is a graphical representation showing _in vivo_ CTL killing of SIVgag overlapping peptide-pulsed cells. Two weeks after the FPV-boost (week 10), 3 equal PBMC populations were labelled with SNARF (2.5µM) or CFSE (2.5 µM or 0.25 µM) and were pulsed with SIVpol, nef or gag overlapping peptide pools (OPAL), respectively.

30   Blood sampled at 5 min, and at 4 and 16 h post-OPAL infusion was RBC-lysed and 10^6 lymphocyte events were acquired by flow cytometry. At 5 min, all 3 populations of

- 7 -

labelled PBMC are of relatively equal numbers. By 4 and 16 hours, 2xDNA/FPV-immunised monkey H20 displayed 27.3% and 76.0% clearance of SIVgag-pulsed PBMC with respect to SIVnef-pulsed PBMC, respectively, whereas no SIVgag-specific killing was observed in control-immunised monkey E20. Note that less events were collected at
5   4 h than 16 h.

Figure 7 is a graphical representation showing vigorous killing of SIVgag- and SIVpol-pulsed PBMC following SHIV challenge. Two weeks after SHIV challenge (week 20), equal PBMC populations were labelled with SNARF (5μM) or CFSE (6μM or 2.5 μM) and were pulsed with SIVpol, no peptide, or SIVgag overlapping peptide pools
10   (OPAL), respectively. 10^6 RBC-lysed lymphocyte events were acquired by flow cytometry. 2xDNA/FPV-immunised monkeys H20 and H21, Displayed 92.3% and 98.3% killing of SIVgag-pulsed PBMC. These animals received 2 separate infusions of SIVpol- pulsed PBMC, furthermore displaying >99% SIVpol-specific killing. Previously CFSE-labelled PBMC were accounted for by flow cytometric analysis of 10^6 lymphocytes immediately
15   prior to OPAL-infusion (not shown).

Figure 8 is a photographic representation showing a boost in T-cell immunogenicity 1 week following OPAL-infusion analysed by IFNγ ELISpot. A boost in SIVgag and pol peptide pool responses is evident in 2xDNA/FPV-immunised monkey H21, where as a primed response to SIVpol peptide pool is detected in control-immunised
20   monkey E20 (week 10 shown above).

Figure 9 is a graphical representation depicting INFγ ELISpot analysis 1 week following OPAL infusion at week 10. A boost in T-cell immunogenicity to SIVgag, pol and nef overlapping peptide pools by OPAL infusion at week 10 was analysed 1 week later by ELISpot. Increased responses to SIVgag were detected in all four 2xDNA/FPV-
25   immunised animals. Increased SIVpol responses were present in the 2xDNA/FPV-immunised monkeys, H20 and H21 (monkeys B00 and H8 did not receive any pol-pulsed PBMC), and in one control-immunised monkey, E20. No responses to SIVnef were primed in any animals. *IFNγ spots in monkeys E20 (prior to OPAL infusion) and B00 (post-OPAL infusion) were excluded due to ELISpot developmental problems.

30   Figure 10 is a graphical representation showing INFγ ELISpot analysis 1 week following OPAL infusion at week 15. A boost in T-cell immunogenicity to SIVgag, pol, nef and HIV-1env overlapping peptide pools by OPAL infusion at week 15 was analysed 1

- 8 -

week later by INFγ ELISpot. Increased responses to SIVgag were detected in all four 2xDNA/FPV-immunised animals. SIVpol responses were marginally increased (or primed) in monkeys, E22, B00, H20 and H21. Increased responses to WI SIV were evident in all animals, whereas no responses were detected for SIVnef or HIV-env in any animals.

5   Figure 11 is a graphical representation depicting mean INFγ ELISpot of immunogenicity of OPAL infusion. Mean INFγ ELISpot responses to (A) SIVgag and (B) SIVpol overlapping peptide pool of control- and 2xDNA/FPV-immunised animals receiving OPAL infusions (bold) were compared to animals receiving equivalent immunisations but no OPAL infusions, before an after the OPAL infusions given at weeks 10   10 and 15 following the immunisation. For the comparison of SIVpol-specific responses, 2xDNA/FPV-immunised animals were grouped based on receiving either 1 (B00 and H8) or 2 (H20 and H21) doses of pol-OPAL infusions.

            Figure 12 is a graphical representation showing the outcome of SHIV intrarectal challenge. At week 18 all control-and 2xDNA/FPV-immunised macaques were challenged 15   intrarectally with SHIV$_{mn229}$ and were assessed for plasma SHIV RNA viral load and CD4+ T cell count over the course of the infection. Recipients of OPAL infusion were compared to their respective immunised non-OPAL recipients. Group comparisons indicate mean ± SE. 2xDNA/FPV-immunised macaques receiving OPAL infusions were further grouped based on receiving either 1or 2 separate doses of pol-pulsed PBMC (B00 20   & H8, and H20 & H21, respectively).

            Figure 13 is a diagrammatic representation showing one embodiment of an HIV-1 consensus B clade Gag peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 124 is 12 amino acids in length. 25   The full-length Gag sequence is modified from the HIV sequence database.

            Figure 14 is a diagrammatic representation showing one embodiment of an HIV-1 consensus B clade Nef peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 49 is 14 amino acids in length. 30   The full-length Nef sequence is modified from the HIV sequence database.

            Figure 15 is a diagrammatic representation showing one embodiment of an HIV-1 consensus B clade Pol peptide pool sequence. Each peptide is 15 amino acids in length and

overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 248 is 14 amino acids in length. The full-length Pol sequence is modified from the HIV sequence database HIV-1.

5   Figure 16 is a diagrammatic representation showing one embodiment of an HIV-1 consensus B clade Rev peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 27 is 13 amino acids in length. The full-length Rev sequence is modified from the HIV sequence database.

10   Figure 17 is a diagrammatic representation showing one embodiment of an HIV-1 consensus B clade Tat peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 24 is 14 amino acids in length. The full-length Tat sequence is modified from the HIV sequence database.

15   Figure 18 is a diagrammatic representation showing one embodiment of an HIV-1 consensus B clade Vif peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 46 is 12 amino acids in length. The full-length Vif sequence is modified from the HIV sequence database.

20   Figure 19 is a diagrammatic representation showing one embodiment of an HIV-1 consensus B clade Vpr peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 22 is 12 amino acids in length. The full-length Vpr sequence is modified from the HIV sequence database.

25   Figure 20 is a diagrammatic representation showing one embodiment of an HIV-1 consensus B clade Vpu peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 18 is 13 amino acids in length. The full-length Vpu sequence is modified from the HIV sequence database.

30   Figure 21 is a diagrammatic representation showing one embodiment of a peptide pool sequence of HCV 1a H77. Each peptide is 18 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide couples 25 & 26, 153 & 154, 220 & 221, 239

- 10 -

& 240, 242 & 243, 244 & 245, 345 & 346 are divided into 15- and 14-mers due to problematic sequences of the original 18-mer peptide. The full-length HCV 1a H77 sequence is modified from the HCV sequence database

- 11 -

DETAILED DESCRIPTION OF THE INVENTION

1. *Definitions*

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred methods and materials are described. For the purposes of the present invention, the following terms are defined below.

The articles "*a*" and "*an*" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

The term "*about*" is used herein to refer to conditions (e.g., amounts, concentrations, time etc) that vary by as much as 30%, preferably by as much as 20%, and more preferably by as much as 10% to a specified condition.

By "*antigen*" is meant all, or part of, a protein, peptide, or other molecule or macromolecule capable of eliciting an immune response in a vertebrate animal, preferably a mammal. Such antigens are also reactive with antibodies from animals immunised with said protein, peptide, or other molecule or macromolecule.

By "*antigen-binding molecule*" is meant a molecule that has binding affinity for a target antigen. It will be understood that this term extends to immunoglobulins, immunoglobulin fragments and non-immunoglobulin derived protein frameworks that exhibit antigen-binding activity.

By "*autologous*" is meant something (e.g., cells, tissues etc) derived from the same organism.

The term "*allogeneic*" as used herein refers to cells, tissues, organisms etc that are of different genetic constitution.

Throughout this specification, unless the context requires otherwise, the words "*comprise*", "*comprises*" and "*comprising*" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements.

- 12 -

By "*corresponds to*" or "*corresponding to*" is meant a polynucleotide (a) having a nucleotide sequence that is substantially identical or complementary to all or a portion of a reference polynucleotide sequence or (b) encoding an amino acid sequence identical to an amino acid sequence in a peptide or protein. This phrase also includes within its scope a

5   peptide or polypeptide having an amino acid sequence that is substantially identical or similar to a sequence of amino acids in a reference peptide or protein.

As used herein, the terms "*culturing*", "*culture*" and the like refer to the set of procedures used *in vitro* where a population of cells (or a single cell) is incubated under conditions which have been shown to support the growth or maintenance of the cells *in*

10   *vitro*. The art recognises a wide number of formats, media, temperature ranges, gas concentrations etc. which need to be defined in a culture system. The parameters will vary based on the format selected and the specific needs of the individual who practices the methods herein disclosed. However, it is recognised that the determination of culture parameters is routine in nature.

15   By "*effective amount*", in the context of modulating an immune response or treating or preventing a disease or condition, is meant the administration of that amount of composition to an individual in need thereof, either in a single dose or as part of a series, that is effective for that modulation, treatment or prevention. The effective amount will vary depending upon the health and physical condition of the individual to be treated, the

20   taxonomic group of individual to be treated, the formulation of the composition, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

By "*expression vector*" is meant any autonomous genetic element capable of directing the synthesis of a protein encoded by the vector. Such expression vectors are

25   known by practitioners in the art.

The term "*gene*" as used herein refers to any and all discrete coding regions of the cell's genome, as well as associated non-coding and regulatory regions. The gene is also intended to mean the open reading frame encoding specific polypeptides, introns, and adjacent 5' and 3' non-coding nucleotide sequences involved in the regulation of

30   expression. In this regard, the gene may further comprise control signals such as promoters, enhancers, termination and/or polyadenylation signals that are naturally associated with a given gene, or heterologous control signals. The DNA sequences may be

cDNA or genomic DNA or a fragment thereof. The gene may be introduced into an appropriate vector for extrachromosomal maintenance or for integration into the host.

Reference herein to *"immuno-interactive"* includes reference to any interaction, reaction, or other form of association between molecules and in particular where one of the molecules is, or mimics, a component of the immune system.

By *"isolated"* is meant material that is substantially or essentially free from components that normally accompany it in its native state.

By *"modulating"* is meant increasing or decreasing, either directly or indirectly, the immune response of an individual.

The term *"operably connected"* or *"operably linked"* as used herein means placing a structural gene under the regulatory control of a promoter, which then controls the transcription and optionally translation of the gene. In the construction of heterologous promoter/structural gene combinations, it is generally preferred to position the genetic sequence or promoter at a distance from the gene transcription start site that is approximately the same as the distance between that genetic sequence or promoter and the gene it controls in its natural setting; *i.e.* the gene from which the genetic sequence or promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting; *i.e.* the genes from which it is derived.

The term *"patient"* refers to patients of mammalian, especially human, or other animal origin and includes any individual it is desired to examine or treat using the methods of the invention. However, it will be understood that *"patient"* does not imply that symptoms are present. Suitable animals that fall within the scope of the invention include, but are not restricted to, primates, livestock animals (e.g., sheep, cows, horses, donkeys, pigs), laboratory test animals (e.g., rabbits, mice, rats, guinea pigs, hamsters), companion animals (e.g., cats, dogs) and captive wild animals (e.g., foxes, deer, dingoes, reptiles, avians, fish).

By *"pharmaceutically-acceptable carrier"* is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in topical or systemic administration.

- 14 -

The term *"polynucleotide"* or *"nucleic acid"* as used herein designates mRNA, RNA, cRNA, cDNA or DNA. The term typically refers to oligonucleotides greater than 30 nucleotides in length.

5   *"Polypeptide"*, *"peptide"* and *"protein"* are used interchangeably herein to refer to a polymer of amino acid residues and to variants and synthetic analogues of the same. Thus, these terms apply to amino acid polymers in which one or more amino acid residues is a synthetic non-naturally occurring amino acid, such as a chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally-occurring amino acid polymers.

10   Reference herein to a *"promoter"* is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (*i.e.* upstream activating sequences, enhancers and silencers) which alter gene expression in response to

15   developmental and/or environmental stimuli, or in a tissue-specific or cell-type-specific manner. A promoter is usually, but not necessarily, positioned upstream or 5', of a structural gene, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene. Preferred promoters according to the invention may contain additional copies of

20   one or more specific regulatory elements to further enhance expression in a cell, and/or to alter the timing of expression of a structural gene to which it is operably connected.

The term *"purified peptide"* means that the peptide is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the peptide is derived, or substantially free from chemical precursors or other chemicals when

25   chemically synthesised. "Substantially free" means that a preparation of a peptide of the invention is at least 10% pure. In certain embodiments, the preparation of peptide has less than about 30%, 25%, 20%, 15%, 10% and desirably 5% (by dry weight), of non-peptide protein (also referred to herein as a "contaminating protein"), or of chemical precursors or non-peptide chemicals. The invention includes isolated or purified preparations of at least

30   0.01, 0.1, 1.0, and 10 milligrams in dry weight.

The term *"recombinant polynucleotide"* as used herein refers to a polynucleotide formed *in vitro* by the manipulation of nucleic acid into a form not normally found in

nature. For example, the recombinant polynucleotide may be in the form of an expression vector. Generally, such expression vectors include transcriptional and translational regulatory nucleic acid operably linked to the nucleotide sequence.

5   By "*recombinant polypeptide*" is meant a polypeptide made using recombinant techniques, *i.e.*, through the expression of a recombinant polynucleotide.

By "*reporter molecule*" as used in the present specification is meant a molecule that, by its chemical nature, provides an analytically identifiable signal that allows the detection of a complex comprising an antigen-binding molecule and its target antigen. The term "reporter molecule" also extends to use of cell agglutination or inhibition of

10   agglutination such as red blood cells on latex beads, and the like.

The term "*sequence identity*" as used herein refers to the extent that sequences are identical on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "*percentage of sequence identity*" is calculated by comparing two optimally aligned sequences over the window of comparison, determining

15   the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, I) or the identical amino acid residue (e.g., Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (*i.e.*, the window size), and multiplying the result

20   by 100 to yield the percentage of sequence identity. For the purposes of the present invention, "*sequence identity*" will be understood to mean the "match percentage" calculated by the DNASIS computer program (Version 2.5 for windows; available from Hitachi Software engineering Co., Ltd., South San Francisco, California, USA) using standard defaults as used in the reference manual accompanying the software.

25   "*Similarity*" refers to the percentage number of amino acids that are identical or constitute conservative substitutions as defined in Table B *infra*. Similarity may be determined using sequence comparison programs such as GAP (Deveraux *et al.* 1984, *Nucleic Acids Research* 12, 387-395). In this way, sequences of a similar or substantially different length to those cited herein might be compared by insertion of gaps into the

30   alignment, such gaps being determined, for example, by the comparison algorithm used by GAP.

- 16 -

Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity" and "substantial identity". A *"reference sequence"* is at least 12 but frequently 15 to 18 and often at least 25 monomer

5   units, inclusive of nucleotides and amino acid residues, in length. Because two polynucleotides may each comprise (1) a sequence (*i.e.*, only a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of

10   the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A *"comparison window"* refers to a conceptual segment of at least 6 contiguous positions, usually about 50 to about 100, more usually about 100 to about 150 in which a sequence is compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. The comparison

15   window may comprise additions or deletions (*i.e.*, gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerised implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release

20   7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (*i.e.*, resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as for example disclosed by Altschul *et al.*, 1997, *Nucl. Acids Res.* 25:3389. A detailed discussion of sequence analysis can be

25   found in Unit 19.3 of Ausubel *et al.*, "Current Protocols in Molecular Biology", John Wiley & Sons Inc, 1994-1998, Chapter 15.

By *"substantially purified population"* and the like is meant that greater than about 80%, usually greater than about 90%, more usually greater than about 95%, typically greater than about 98%, and more typically greater than about 99% of the cells in the

30   population are antigen-presenting cells of a chosen type.

By *"vector"* is meant a nucleic acid molecule, preferably a DNA molecule derived, for example, from a plasmid, bacteriophage, or plant virus, into which a nucleic acid sequence may be inserted or cloned. A vector preferably contains one or more unique

- 17 -

restriction sites and may be capable of autonomous replication in a defined host cell including a target cell or tissue or a progenitor cell or tissue thereof, or be integrable with the genome of the defined host such that the cloned sequence is reproducible. Accordingly, the vector may be an autonomously replicating vector, *i.e.*, a vector that exists as an
5   extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a linear or closed circular plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s)
10   into which it has been integrated. A vector system may comprise a single vector or plasmid, two or more vectors or plasmids, which together contain the total DNA to be introduced into the genome of the host cell, or a transposon. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vector may also include a selection marker such as an antibiotic
15   resistance gene that can be used for selection of suitable transformants.

2.   *Immunomodulating sets of overlapping peptides*

The present invention is predicated in part on the discovery that antigen-presenting cells contacted *ex vivo* with a set of overlapping peptides spanning a viral polypeptide antigen of interest (also referred to herein as Overlapping Peptide-pulsed
20   Autologous ceLls, OPAL) are effective in producing a strong immunogenic response that protects against subsequent viral challenge in an outbred population, without prior knowledge of the epitopes of the antigen. Since antigen-presenting cells form a significant part of the circulatory system, it is proposed that systemic delivery of the overlapping peptides *per se* will produce a similar protective effect. Accordingly, the present invention
25   broadly provides a set of peptides for modulating an immune response to a polypeptide of interest, wherein individual peptides comprise different portions of an amino acid sequence corresponding to the polypeptide of interest and display partial sequence identity or similarity to at least one other peptide of the set.

The partial sequence identity or similarity is typically contained at one or both
30   ends of an individual peptide. In one embodiment, there are at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 40, 50 contiguous amino acid residues at one or both ends of an individual peptide, whose sequence is identical or similar to an amino acid sequence

- 18 -

contained within at least one other of the peptides. In an alternate embodiment, there are less than 500, 100, 50, 40, 30 contiguous amino acid residues at one or both ends of an individual peptide, whose sequence is identical or similar to an amino acid sequence contained within at least one other of the peptides. Such 'sequence overlap' is

5 advantageous to prevent or otherwise reduce the loss of any potential epitopes contained within a polypeptide of interest. One example, the sequence overlap is 11 amino acid residues.

Typically, when peptides have partial sequence similarity, their sequences will usually differ by one or more conserved and/or non-conserved amino acid substitutions.

10 Exemplary conservative substitutions are listed in the following table.

TABLE A

Original Residue	Exemplary Substitutions	Original Residue	Exemplary Substitutions
Ala	Ser	Leu	Ile, Val
Arg	Lys	Lys	Arg, Gln, Glu
Asn	Gln, His	Met	Leu, Ile,
Asp	Glu	Phe	Met, Leu, Tyr
Cys	Ser	Ser	Thr
Gln	Asn	Thr	Ser
Glu	Asp	Trp	Tyr
Gly	Pro	Tyr	Trp, Phe
His	Asn, Gln	Val	Ile, Leu
Ile	Leu, Val		

Conserved or non-conserved substitutions may correspond to polymorphisms in a polypeptide of interest. Polymorphic polypeptides are expressed by various pathogenic

15 organisms and cancers. For example, the polymorphic polypeptides may be expressed by different viral strains or clades or by cancers in different individuals.

The peptides of the invention may be of any suitable size that can be utilised to elicit an immune response to a polypeptide of interest. A number of factors can influence

the choice of peptide size. For example, the size of a peptide can be chosen such that it includes, or corresponds to the size of, T cell epitopes and/or B cell epitopes, and their processing requirements. Practitioners in the art will recognise that class I-restricted T cell epitopes are typically between 8 and 10 amino acid residues in length and if placed next to

5   unnatural flanking residues, such epitopes can generally require 2 to 3 natural flanking amino acid residues to ensure that they are efficiently processed and presented. Class II-restricted T cell epitopes usually range between 12 and 25 amino acid residues in length and may not require natural flanking residues for efficient proteolytic processing although it is believed that natural flanking residues may play a role. Another important feature of

10   class II-restricted epitopes is that they generally contain a core of 9-10 amino acid residues in the middle which bind specifically to class II MHC molecules with flanking sequences either side of this core stabilising binding by associating with conserved structures on either side of class II MHC antigens in a sequence independent manner. Thus the functional region of class II-restricted epitopes is typically less than about 15 amino acid

15   residues long. The size of linear B cell epitopes and the factors effecting their processing, like class II-restricted epitopes, are quite variable although such epitopes are frequently smaller in size than 15 amino acid residues. From the foregoing, it is advantageous, but not essential, that the size of the peptide is at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30 amino acid residues. Suitably, the size of the peptide is no more than about 500, 200, 100,

20   80, 60, 50, 40 amino acid residues. In one embodiment, the size of the peptide is large enough to minimise loss of T cell and/or B cell epitopes. In another embodiment, the size of the peptide is sufficient for presentation by an antigen-presenting cell of a T cell and/or a B cell epitope contained within the peptide. In one example of this embodiment, the size of the peptide is about 15 amino acid residues.

25   The polypeptide of interest is suitably a disease- or condition-associated antigen. In one embodiment, the disease- or condition-associated antigen is produced by a pathogenic organism. Pathogenic organisms include, but are not restricted to, yeast, viruses, bacteria, helminths, protozoans and mycoplasmas. Natural hosts for such organisms may be selected from mammals, avians and fish. In one embodiment, the

30   pathogenic organism is a virus. Suitable viruses include RNA viruses or DNA viruses. For example, viruses may be selected from human immunodeficiency virus (HIV), herpes virus, cytomegalovirus, rabies virus, influenza virus, hepatitis A virus, hepatitis B virus, hepatitis C virus, Sendai virus, Rous sarcoma virus, feline leukemia virus, Reo virus, polio

- 20 -

virus, human serum parvo-like virus, simian virus 40, respiratory syncytial virus, mouse mammary tumour virus, Varicella-Zoster virus, Dengue virus, rubella virus, measles virus, adenovirus, human T-cell leukemia viruses, Epstein-Barr virus, cytomegalovirus, flavivirus such as Japanese encephalitis virus, arboviruses such as bunyaviruses, reoviruses,
5   rhabdoviruses and togaviruses, murine leukemia virus, mumps virus, vesicular stomatitis virus, Sindbis virus, lymphocytic choriomeningitis virus, wart virus, blue tongue virus, Newcastle Disease virus, Infectious Bursal Disease virus, fowl pox virus, sheep pox virus, Laryngotracheitis virus, Infectious Bronchitis of poultry virus, Gumboro Winterfield virus, Infectious Bronchitis and Rinderpest virus. In one example of the present invention, the
10   virus is HIV and the polypeptide of interest is suitably selected from env, gag, pol, vif, vpr, tat, rev, vpu and nef, or combination thereof.

In another embodiment, the pathogenic organism is a bacterium, which includes, but is not restricted to, *Escherichia* species (e.g., *Escherichia coli*), Neisseria species (e.g., *Neisseria gonorrhoeae*, *Neisseria meningitidis*), *Meningococcal* species (e.g.,),
15   *Haemophilus* species (e.g., *Hemophilus influenzae* B), *Pneumococcus* species, *Salmonella* species (e.g.,), *Streptococcal* species (e.g., *Streptococcus pyogenes*, *Streptococcus agalactiae*), *Legionella* species (e.g., *Legionella pneumophilia*), *Pseudomonas* species (e.g., *Pseudomonas aeroginosa*) and *Mycobacterium* species (e.g., *Mycobacterium leprae*, *Mycobacterium tuberculosis*), *Treponema* species (e.g., *Treponema pallidum*), Lyme
20   disease spirochetes, *Brucella* species (e.g., *Brucella abortus*), and Tetanus toxin.

In yet another embodiment, the pathogenic organism is a protozoan which is suitably selected from the group consisting of *Plasmodium* species (e.g., *Plasmodium falciparum*, *Plasmodium vivax*), *Toxoplasma* species (e.g., *Toxoplasma gondii*), *Trypanosoma* species (e.g., *Trypanosoma rangeli*, *Trypanosoma cruzi*, *Trypanosoma*
25   *rhodesiensei*, *Trypanosoma brucei*), *Schistosoma* species (e.g., *Schistosoma mansoni*, *Schistosoma japanicum*), *Giardia* species, *Babesia* species (e.g., *Babesia bovis*), *Elmeria tenella*, *Onchocerca volvulus*, *Leishmania* species (e.g., *Leishmania tropica*), *Trichinella spiralis*, *Onchocerca volvulus*, *Theileria parva*, *Taenia hydatigena*, *Taenia ovis*, *Taenia saginata*, *Echinococcus granulosus* and *Mesocestoides corti*.

30   In still another embodiment, the pathogenic organism is a mycoplasma which includes, but is not limited to, *Mycoplasma arthritidis*, *Mycoplasma hyorhinis*, Mycoplasma *orale*, *Mycoplasma arginini*, *Acholeplasma laidlawii*, *Mycoplasma salivarum*, and *Mycoplasma pneumoniae*.

- 21 -

Alternatively, the disease- or condition-associated antigen is an antigen expressed by a cancer or tumour, which is suitably selected from melanoma, lung cancer, breast cancer, cervical cancer, prostate cancer, colon cancer, pancreatic cancer, stomach cancer, bladder cancer, kidney cancer, post transplant lymphoproliferative disease (PTLD),

5   Hodgkin's Lymphoma and the like. In one embodiment, the cancer or tumour relates to melanoma. In this embodiment, the polypeptide of interest can be a melanocyte differentiation antigen which is suitably selected from gp100, MART, TRP-1, Tyros, TRP2, MC1R, MUC1F, MUC1R or a combination thereof. Alternatively, the polypeptide of interest is a melanoma-specific antigen which is suitably selected from BAGE, GAGE-

10   1, gp100In4, MAGE-1, MAGE-3, PRAME, TRP2IN2, NYNSO1a, NYNSO1b, LAGE1 or a combination thereof.

In one example of the present invention, the size of individual peptides is about 14 or 15 amino acid residues and the sequence overlap at one or both ends of an individual peptide is about 11 amino acid residues. However, it will be understood that other suitable

15   peptide sizes and sequence overlap sizes are contemplated by the present invention, which can be readily ascertained by persons of skill in the art.

It is advantageous but not necessary to utilise the entire sequence of a polypeptide of interest for producing a set of overlapping peptides. Typically, at least 30%, 40%, 50%, 60%, 70%, 80% 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% of the sequence

20   corresponding to a polypeptide of interest is used to produce the overlapping peptides of the invention. However, it will be understood that the more sequence information from a polypeptide of interest that is utilised to produce the overlapping peptides, the greater the population coverage will be of the overlapping peptides as an immunogen. Suitably, no sequence information from the polypeptide of interest is excluded (e.g., because of an

25   apparent lack of immunological epitopes). If required, variable and/or hypervariable sequences within a polypeptide of interest can be included in the construction of an overlapping set of peptides. In this embodiment, multiple peptides are synthesised to cover the variation in amino acid residue at individual positions of a polypeptide of interest. Peptide sequences may include additional sequences that are not derived from a

30   polypeptide of interest. These additional sequences may have various functions, including improving solubility or immunogenicity or facilitating purification. Typically, such additional sequences are contained at one or both ends of a respective peptide.

- 22 -

Persons of skill in the art will appreciate that when preparing a set of overlapping peptides according to the invention, it may be advantageous to use sequence information from a plurality of different polypeptides produced by a pathogenic organism or expressed in a cancer. Accordingly, in certain embodiments, at least 2, 3, 4, 5, 6, 7, 9, 10, 15, 20 other
5   sets of peptides are used for the production of the immunomodulating compositions of the invention, wherein the sequences of a respective other set of peptides are derived from a distinct polypeptide of interest and wherein individual peptides of the respective other set display partial sequence identity or similarity to at least one other peptide of a corresponding set of peptides. It is advantageous in this respect to utilise as many
10   polypeptides as possible from, or in relation to, a particular source in the construction of sets of overlapping peptides. Suitably, at least about 30%, 40%, 50%, 60%, 70%, 80% 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, and desirably 100%, of the polypeptides expressed by the source is used in the construction of the corresponding sets of overlapping peptides. Exemplary viral polypeptides that can be used for such
15   construction include, but are not restricted to, latent polypeptides, regulatory polypeptides or polypeptides expressed early during their replication cycle. Suitably, polypeptides from a protozoan, bacterium, mycoplasma, fungus or helminth include, but are not restricted to, secretory polypeptides, regulatory polypeptides and polypeptides expressed on the surface of these organisms. Polypeptides from a cancer or tumour, which can be used for the
20   construction of overlapping peptide sets, are suitably cancer-specific polypeptides.

Representative overlapping peptide sets for modulating the immune response to simian immunodeficiency virus (SIV) and/or the chimeric SIV-HIV-1 (SHIV) can be based on one or more polypeptides selected from SIV gag, pol, nef or SHIV env as for example presented in Figures 1 to 4. Exemplary overlapping peptide sets for modulating
25   the immune response to HIV-1 can be based on one or more polypeptides selected from HIV Gag, Nef, Pol, Rev, Tat, Vif, Vpr and Vpu as for example set forth in Figures 13 to 20. Exemplary overlapping peptide sets for modulating the immune response to HCV 1a can be based on the HCV 1a H77 polyprotein sequence as for example set forth in Figure 21.

30   The overlapping peptide sets of the invention may be prepared by any suitable procedure known to those of skill in the art. For example, the peptide sets can be synthesised conveniently using solution synthesis or solid phase synthesis as described, for example, in Chapter 9 of Atherton and Shephard (1989, Solid Phase Peptide Synthesis: A

Practical Approach. IRL Press, Oxford) and in Roberge *et al* (1995, *Science* 269: 202). Syntheses may employ, for example, either *t*-butyloxycarbonyl (*t*-Boc) or 9-fluorenylmethyloxycarbonyl (Fmoc) chemistries (see Chapter 9.1, of Coligan *et al.*, CURRENT PROTOCOLS IN PROTEIN SCIENCE, John Wiley & Sons, Inc. 1995-1997; Stewart and Young, 1984, Solid Phase Peptide Synthesis, 2nd ed. Pierce Chemical Co., Rockford, Ill; and Atherton and Shephard, *supra*).

Alternatively, individual peptides may be prepared by a procedure including the steps of: (a) preparing a synthetic construct including a synthetic polynucleotide encoding an individual peptide of an overlapping set of peptides, wherein the synthetic polynucleotide is operably linked to a regulatory polynucleotide; (b) introducing the synthetic construct into a suitable host cell; (c) culturing the host cell to express the synthetic polynucleotide; and (d) isolating the individual peptide. The synthetic construct is preferably in the form of an expression vector. For example, the expression vector can be a self-replicating extra-chromosomal vector such as a plasmid, or a vector that integrates into a host genome. Typically, the regulatory polynucleotide includes, but is not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and termination sequences, and enhancer or activator sequences. Constitutive or inducible promoters as known in the art are contemplated by the invention. The promoters may be either naturally occurring promoters, or hybrid promoters that combine elements of more than one promoter. The regulatory polynucleotide will generally be appropriate for the host cell used for expression. Numerous types of appropriate expression vectors and suitable regulatory polynucleotides are known in the art for a variety of host cells. In certain embodiments, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selection genes are well known in the art and will vary with the host cell used. In other embodiments, the expression vector also includes a nucleic acid sequence that codes for a fusion partner so that an individual peptide is expressed as a fusion polypeptide with the fusion partner. The main advantage of fusion partners is that they assist identification and/or purification of the fusion polypeptide. Exemplary fusion partners include, but are not limited to, glutathione-S-transferase (GST), Fc portion of human IgG, maltose binding protein (MBP) and hexahistidine (HIS$_6$), which are particularly useful for isolation of the fusion polypeptide by affinity chromatography. For the purposes of fusion polypeptide purification by affinity chromatography, relevant matrices for affinity

- 24 -

chromatography are glutathione-, amylose-, and nickel- or cobalt-conjugated resins respectively. Many such matrices are available in "kit" form, such as the QIAexpress™ system (Qiagen) useful with (HIS6) fusion partners and the Pharmacia GST purification system. In a preferred embodiment, the recombinant polynucleotide is expressed in the
5   commercial vector pFLAG™. Advantageously, the fusion partners also have protease cleavage sites, such as for Factor X_a, Thrombin and inteins (protein introns), which allow the relevant protease to partially digest the fusion polypeptide of the invention and thereby liberate the recombinant polypeptide of the invention therefrom. The liberated peptide can then be isolated from the fusion partner by subsequent chromatographic separation. Fusion
10   partners according to the invention also include within their scope "epitope tags", which are usually short peptide sequences for which a specific antibody is available. Well known examples of epitope tags for which specific monoclonal antibodies are readily available include c-Myc, influenza virus, haemagglutinin and FLAG tags.

The step of introducing the synthetic construct into the host cell may be achieved
15   using any suitable technique including transfection, and transformation, the choice of which will be dependent on the host cell employed. Such methods are well known to those of skill in the art. The peptides of the invention may be produced by culturing a host cell transformed with the synthetic construct. The conditions appropriate for protein expression will vary with the choice of expression vector and the host cell. This is easily ascertained
20   by one skilled in the art through routine experimentation. Suitable host cells for expression may be prokaryotic or eukaryotic. One preferred host cell for expression of a polypeptide according to the invention is a bacterium. The bacterium used may be *Escherichia coli*. Alternatively, the host cell may be an insect cell such as, for example, *SF9* cells that may be utilised with a baculovirus expression system.

25   The amino acids of the peptides can be any non-naturally occurring or any naturally occurring amino acid. Examples of unnatural amino acids and derivatives during peptide synthesis include but are not limited to, use of 4-amino butyric acid, 6-aminohexanoic acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 4-amino-3-hydroxy-6-methylheptanoic acid, t-butylglycine, norleucine, norvaline, phenylglycine, ornithine,
30   sarcosine, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acids contemplated by the present invention is shown in TABLE B.

TABLE B

Non-conventional amino acid	Non-conventional amino acid
α-aminobutyric acid	L-N-methylalanine
α-amino-α-methylbutyrate	L-N-methylarginine
aminocyclopropane-carboxylate	L-N-methylasparagine
aminoisobutyric acid	L-N-methylaspartic acid
aminonorbornyl-carboxylate	L-N-methylcysteine
cyclohexylalanine	L-N-methylglutamine
cyclopentylalanine	L-N-methylglutamic acid
L-N-methylisoleucine	L-N-methylhistidine
D-alanine	L-N-methylleucine
D-arginine	L-N-methyllysine
D-aspartic acid	L-N-methylmethionine
D-cysteine	L-N-methylnorleucine
D-glutamate	L-N-methylnorvaline
D-glutamic acid	L-N-methylornithine
D-histidine	L-N-methylphenylalanine
D-isoleucine	L-N-methylproline
D-leucine	L-N-medlylserine
D-lysine	L-N-methylthreonine
D-methionine	L-N-methyltryptophan
D-ornithine	L-N-methyltyrosine
D-phenylalanine	L-N-methylvaline
D-proline	L-N-methylethylglycine
D-serine	L-N-methyl-t-butylglycine
D-threonine	L-norleucine

- 26 -

Non-conventional amino acid	Non-conventional amino acid
D-tryptophan.	L-norvaline
D-tyrosine	α-methyl-aminoisobutyrate
D-valine	α-methyl-γ-aminobutyrate
D-α-methylalanine	α-methylcyclohexylalanine
D-α-methylarginine	α-methylcylcopentylalanine
D-α-methylasparagine	α-methyl-α-napthylalanine
D-α-methylaspartate	α-methylpenicillamine
D-α-methylcysteine	N-(4-aminobutyl)glycine
D-α-methylglutamine	N-(2-aminoethyl)glycine
D-α-methylhistidine	N-(3-aminopropyl)glycine
D-α-methylisoleucine	N-amino-α-methylbutyrate
D-α-methylleucine	α-napthylalanine
D-α-methyllysine	N-benzylglycine
D-α-methylmethionine	N-(2-carbamylediyl)glycine
D-α-methylornithiine	N-(carbamylmethyl)glycine
D-α-methylphenylalanine	N-(2-carboxyethyl)glycine
D-α-methylproline	N-(carboxymethyl)glycine
D-α-methylserine	N-cyclobutylglycine
D-α-methylthreonine	N-cycloheptylglycine
D-α-methyltryptophan	N-cyclohexylglycine
D-α-methyltyrosine	N-cyclodecylglycine
L-α-methylleucine	L-α-methyllysine
L-α-methylmethionine	L-α-methylnorleucine
L-α-methylnorvatine	L-α-methylornithine
L-α-methylphenylalanine	L-α-methylproline

- 27 -

Non-conventional amino acid	Non-conventional amino acid
L-α-methylserine	L-α-methylthreonine
L-α-methyltryptophan	L-α-methyltyrosine
L-α-methylvaline	L-N-methylhomophenylalanine
N-(N-(2,2-diphenylethyl carbamylmethyl)glycine	N-(N-(3,3-diphenylpropyl carbamylmethyl)glycine
1-carboxy-1-(2,2-diphenyl-ethyl amino)cyclopropane	

The invention also contemplates modifying the peptides of the invention using ordinary molecular biological techniques so as to alter their resistance to proteolytic degradation or to optimise solubility properties or to render them more suitable as an
5   immunogenic agent.

3. Antigen-presenting cell embodiments

The present invention is predicated in part on the determination that antigen-presenting cells which have been contacted with overlapping peptide sets as described in Section 2 are potent modulators of immune responses and are especially useful for raising
10   strong immunogenic responses that can prevent or ameliorate the symptoms of a disease or condition of interest. Accordingly, the invention provides a process for producing antigen-specific antigen-presenting cells, comprising contacting antigen-presenting cells or their precursors with one or more sets of peptides as broadly described above for a time and under conditions sufficient for the peptides or processed forms thereof to be presented by
15   the antigen-presenting cells or their precursors, and in the case of precursors, culturing the precursors for a time and under conditions sufficient to differentiate antigen-presenting cells from the precursors.

3.1 Sources of antigen-presenting cells

The antigen-presenting cells suitably encompasses both professional and
20   facultative types of antigen-presenting cells. For example, professional antigen-presenting cells include, but are not limited to, macrophages, monocytes, cells of myeloid lineage, including monocytic-granulocytic-DC precursors, marginal zone Kupffer cells, microglia, T cells, B cells Langerhans cells and dendritic cells including interdigitating dendritic cells

- 28 -

and follicular dendritic cells. Examples of facultative antigen-presenting cells include but are not limited to activated T cells, astrocytes, follicular cells, endothelium and fibroblasts. In a preferred embodiment, the antigen-presenting cells are selected from monocytes, macrophages, cells of myeloid lineage, dendritic cells or Langerhans cells.

5   Antigen-presenting cells or their precursors can be isolated by methods known to those of skill in the art. The source of antigen-presenting cell or precursor will differ depending upon the antigen-presenting cell required for modulating a specified immune response. In this context, the antigen-presenting cell can be selected from dendritic cells, macrophages, monocytes and other cells of myeloid lineage. Typically, precursors of
10   antigen-presenting cells can be isolated from any tissue, but are most easily isolated from blood, cord blood or bone marrow (Sorg *et al.*, 2001, *Exp Hematol* **29**: 1289-1294; Zheng *et al.*, 2000, *J Hematother Stem Cell Res* **9**: 453-464). It is also possible to obtain suitable precursors from diseased tissues such as rheumatoid synovial tissue or fluid following biopsy or joint tap (Thomas *et al.*, 1994, *J Immunol* **152**: 2613-2623; Thomas *et al.*, 1994,
15   *J Immunol* **153**: 4016-4028). Other examples include, but are not limited to liver, spleen, heart, kidney, gut and tonsil (Lu *et al.*, 1994, *Transplantation* **64**: 1808-1815; McIlroy *et al.*, 2001, *Blood* **97**: 3470-3477; Vremec *et al.*, 2000, *J Immunol* **164**: 2978-2986; Hart and Fabre, 1981, *J Exp Med* **154**(2): 347-361; Hart and McKenzie, 1988, *J Exp Med* **168**(1): 157-170; Pavli *et al.*, 1990, *Immunology* **70**(1): 40-47).

20   Leukocytes isolated directly from tissue provide a major source of antigen-presenting cell precursors. Typically, these precursors can only differentiate into antigen-presenting cells by culturing in the presence or absence of various growth factors. According to the practice of the present invention, the antigen-presenting cells may be so differentiated from crude mixtures or from partially or substantially purified preparations
25   of precursors. Leukocytes can be conveniently purified from blood or bone marrow by density gradient centrifugation using, for example, Ficoll Hypaque which eliminates neutrophils and red cells (peripheral blood mononuclear cells or PBMCs), or by ammonium chloride lysis of red cells (leukocytes or white blood cells). Many precursors of antigen-presenting cells are present in peripheral blood as non-proliferating monocytes,
30   which can be differentiated into specific antigen-presenting cells, including macrophages and dendritic cells, by culturing in the presence of specific cytokines.

Tissue-derived precursors such as precursors of tissue dendritic cells or of Langerhans cells are typically obtained by mincing tissue (e.g., basal layer of epidermis)

and digesting it with collagenase or dispase followed by density gradient separation, or selection of precursors based on their expression of cell surface markers. For example, Langerhans cell precursors express CD1 molecules as well as HLA-DR and can be purified on this basis.

5   In one embodiment, the antigen-presenting cell precursor is a precursor of macrophages. Generally these precursors can be obtained from monocytes of any source and can be differentiated into macrophages by prolonged incubation in the presence of medium and macrophage colony stimulating factor (M-CSF) (Erickson-Miller *et al.*, 1990, *Int J Cell Cloning* **8**: 346-356; Metcalf and Burgess, 1982, *J Cell Physiol* **111**: 275-283).

10   In another embodiment, the antigen presenting cell precursor is a precursor of Langerhans cells. Usually, Langerhans cells can be generated from human monocytes or CD34$^+$ bone marrow precursors in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-4/TNFα and TGFβ (Geissmann *et al.*, 1998, *J Exp Med* **187**: 961-966; Strobl *et al.*, 1997, *Blood* **90**: 1425-1434 Strobl *et al.*, 1997, *Adv Exp Med*
15   *Biol* **417**: 161-165; Strobl *et al.*, 1996, *J Immunol* **157**: 1499-1507).

In one embodiment, the antigen-presenting cell precursor is a precursor of dendritic cells. Several potential dendritic cell precursors can be obtained from peripheral blood, cord blood or bone marrow. These include monocytes, CD34$^+$ stem cells, granulocytes, CD33$^+$CD11c$^+$ DC precursors, and committed myeloid progenitors –
20   described below.

Monocytes. Monocytes can be purified by adherence to plastic for 1-2 h in the presence of tissue culture medium (e.g., RPMI) and serum (e.g., human or foetal calf serum), or in serum-free medium (Anton *et al.*, 1998, *Scand J Immunol* **47**: 116-121.; Araki *et al.*, 2001, Br *J Haematol* **114**: 681-689; Mackensen *et al.*, 2000, *Int J Cancer* **86**:
25   385-392; Nestle *et al.*, 1998, *Nat Med* **4**: 328-332; Romani *et al.*, 1996, *J Immunol Meth* **196**: 137-151; Thurner *et al.*, 1999, *J Immunol Methods* **223**: 1-15). Monocytes can also be elutriated from peripheral blood (Garderet *et al.*, 2001, *J Hematother Stem Cell Res* **10**: 553-567). Monocytes can also be purified by immunoaffinity techniques, including immunomagnetic selection, flow cytometric sorting or panning (Araki *et al.*, 2001, *supra*;
30   Battye and Shortman, 1991, *Curr. Opin. Immunol.* **3**: 238-241), with anti-CD14 antibodies to obtain CD14^{hi} cells. The numbers (and therefore yield) of circulating monocytes can be enhanced by the *in vivo* use of various cytokines including GM-CSF (Groopman *et al.*,

1987; *N Engl J Med* 317: 593-598; Hill *et al.*, 1995, *J Leukoc Biol* 58: 634-642). Monocytes can be differentiated into dendritic cells by prolonged incubation in the presence of GM-CSF and IL-4 (Romani *et al.*, 1994, *J Exp Med* 180: 83-93; Romani *et al.*, 1996, *supra*). A combination of GM-CSF and IL-4 at a concentration of each at between about 200 to about 2000 U/mL, more preferably between about 500 to about 1000 U/mL and even more preferably between about 800 U/mL (GM-CSF) and 1000 U/mL (IL-4) produces significant quantities of immature dendritic cells, *i.e.*, antigen-capturing phagocytic dendritic cells. Other cytokines which promote differentiation of monocytes into antigen-capturing phagocytic dendritic cells include, for example, IL-13.

CD34$^+$ stem cells. Dendritic cells can also be generated from CD34$^+$ bone marrow derived precursors in the presence of GM-CSF, TNFα ± stem cell factor (SCF, c-kitL), or GM-CSF, IL-4 ± flt3L (Bai *et al.*, 2002, *Int J Oncol* 20: 247-53; Chen *et al.*, 2001, *Clin Immunol* 98: 280-292; Loudovaris *et al.*, 2001, *J Hematother Stem Cell Res* 10: 569-578). CD34$^+$ cells can be derived from a bone marrow aspirate or from blood and can be enriched as for monocytes using, for example, immunomagnetic selection or immunocolumns (Davis *et al.*, 1994, *J Immunol Meth* 175: 247-257). The proportion of CD34$^+$ cells in blood can be enhanced by the *in vivo* use of various cytokines including (most commonly) G-CSF, but also flt3L and progenipoietin (Fleming *et al.*, 2001, *Exp Hematol* 29: 943-951; Pulendran *et al.*, 2000, *J Immunol* 165: 566-572; Robinson *et al.*, 2000, *J Hematother Stem Cell Res* 9: 711-720).

Other myeloid progenitors. DC can be generated from committed early myeloid progenitors in a similar fashion to CD34$^+$ stem cells, in the presence of GM-CSF and IL-4/TNF. Such myeloid precursors infiltrate many tissues in inflammation, including rheumatoid arthritis synovial fluid (Santiago-Schwarz *et al.*, 2001, *J Immunol* 167(3): 1758-68). Expansion of total body myeloid cells including circulating dendritic cell precursors and monocytes, can be achieved with certain cytokines, including flt-3 ligand, granulocyte colony-stimulating factor (G-CSF) or progenipoietin (pro-GP) (Fleming *et al.*, 2001, *supra*; Pulendran *et al.*, 2000, *supra*; Robinson *et al.*, 2000, *supra*). Administration of such cytokines for several days to a human or other mammal would enable much larger numbers of precursors to be derived from peripheral blood or bone marrow for *in vitro* manipulation. Dendritic cells can also be generated from peripheral blood neutrophil precursors in the presence of GM-CSF, IL-4 and TNFα (Kelly *et al.*, 2001, *Cell Mol Biol* (Noisy-le-grand) 47(1): 43-54; Oehler *et al.*, 1998, *J Exp Med.* 187(7):1019-28). It should

- 31 -

be noted that dendritic cells can also be generated, using similar methods, from acute myeloid leukemia cells (Oehler *et al.*, 2000, *Ann Hematol* 79(7): 355-62).

Tissue DC precursors and other sources of APC precursors. Other methods for DC generation exist from, for example, thymic precursors in the presence of IL-3 +/-
5   GM-CSF, and liver DC precursors in the presence of GM-CSF and a collagen matrix. Transformed or immortalised dendritic cell lines may be produced using oncogenes such as *v-myc* as for example described by (Paglia *et al.*, 1993, *J Exp Med* 178(6): 1893-901) or by myb (Banyer and Hapel, 1999, *J Leukoc Biol* 66(2): 217-223; Gonda *et al.*, 1993, *Blood* 82(9): 2813-2822).

10   **Circulating DC precursors.** These have been described in human and mouse peripheral blood. One can also take advantage of particular cell surface markers for identifying suitable dendritic cell precursors. Specifically, various populations of dendritic cell precursors can be identified in blood by the expression of CD11c and the absence or low expression of CD14, CD19, CD56 and CD3 (O'Doherty *et al.*, 1994, *Immunology* 82:
15   487-493; O'Doherty *et al.*, 1993, *J Exp Med* 178: 1067-1078). These cells can also be identified by the cell surface markers CD13 and CD33 (Thomas *et al.*, 1993, *J Immunol* 151(12): 6840-6852). A second subset, which lacks CD14, CD19, CD56 and CD3, known as plasmacytoid dendritic cell precursors, does not express CD11c, but does express CD123 (IL-3R chain) and HLA-DR (Farkas *et al.*, 2001, *Am J Pathol* 159: 237-243;
20   Grouard *et al.*, 1997, *J Exp Med* 185: 1101-1111; Rissoan *et al.*, 1999, *Science* 283: 1183-1186). Most circulating CD11c$^+$ dendritic cell precursors are HLA-DR$^+$, however some precursors may be HLA-DR-. The lack of MHC class II expression has been clearly demonstrated for peripheral blood dendritic cell precursors (del Hoyo *et al.*, 2002, *Nature* 415: 1043-1047).

25   Optionally, CD33$^+$CD14$^{-/lo}$ or CD11c$^+$HLA-DR$^+$, lineage marker-negative dendritic cell precursors described above can be differentiated into more mature antigen-presenting cells by incubation for 18-36 h in culture medium or in monocyte conditioned medium (Thomas *et al.*, 1993, *J Immunol* 151(12): 6840-6852; Thomas and Lipsky, 1994, *J Immunol* 153: 4016-4028; O'Doherty *et al.*, 1993, *supra*). Alternatively, following
30   incubation of peripheral blood non-T cells or unpurified PBMC, the mature peripheral blood dendritic cells are characterised by low density and so can be purified on density gradients, including metrizamide and Nycodenz (Freudenthal and Steinman, 1990, *Proc Natl Acad Sci U S A* 87: 7698-7702; Vremec and Shortman, 1997, *J Immunol* 159: 565-

573), or by specific monoclonal antibodies, such as but not limited to the CMRF-44 mAb (Fearnley *et al.*, 1999, *Blood* 93, 728-736; Vuckovic *et al.*, 1998, *Exp Hematol* 26: 1255-1264). Plasmacytoid dendritic cells can be purified directly from peripheral blood on the basis of cell surface markers, and then incubated in the presence of IL-3 (Grouard *et al.*, 1997, *supra*; Rissoan *et al.*, 1999, *supra*). Alternatively, plasmacytoid DC can be derived from density gradients or CMRF-44 selection of incubated peripheral blood cells as above.

In general, for dendritic cells generated from any precursor, when incubated in the presence of activation factors such as monocyte-derived cytokines, lipopolysaccharide and DNA containing CpG repeats, cytokines such as TNF-α, IL-6, IFN-α, IL-1β, necrotic cells, readherence, whole bacteria, membrane components, RNA or polyIC, immature dendritic cells will become activated (Clark, 2002, *J Leukoc Biol* 71: 388-400; Hacker *et al.*, 2002, *Immunology* 105: 245-251; Kaisho and Akira, 2002, *Biochim Biophys Acta* 1589: 1-13; Koski *et al.*, 2001, *Crit Rev Immunol* 21: 179-189).

Other methods for isolation, expansion and/or maturation of dendritic cells for the purpose of the present invention are described for example by Takamizawa *et al.* (1997, *J Immunol*, 158(5): 2134-2142), Thomas and Lipsky (1994, *J Immunol*, 153(9): 4016-4028), O'Doherty *et al.* (1994, *Immunology*, 82(3): 487-93), Fearnley *et al.* (1997, *Blood*, 89(10): 3708-3716), Weissman *et al.* (1995, *Proc Natl Acad Sci U S A*, 92(3): 826-830), Freudenthal and Steinman (1990, *Proc Natl Acad Sci U S A*, 87(19): 7698-7702), Romani *et al.* (1996, *J Immunol Methods*, 196(2): 137-151), Reddy *et al.* (1997, *Blood*, 90(9): 3640-3646), Thurnher *et al.* (1997, *Exp Hematol*, 25(3): 232-237), Caux *et al.* (1996, *J Exp Med*, 184(2): 695-706; 1996, *Blood*, 87(6): 2376-85), Luft *et al.* (1998, *Exp Hematol*, 26(6): 489-500; 1998, *J Immunol*, 161(4): 1947-1953), Cella *et al.* (1999, *J Exp Med*, 189(5): 821-829; 1997, *Nature*, 388(644): 782-787; 1996, *J Exp Med*, 184(2): 747-572), Ahonen *et al.* (1999, *Cell Immunol*, 197(1): 62-72) and Piemonti *et al.* (1999, *J Immunol*, 162(11): 6473-6481).

In some embodiments, the antigen-presenting cells are in the form of a substantially purified population of cells. In other embodiments, the antigen-presenting cells are in the form of a heterogenous pool of cells, which is suitably selected from blood and peripheral blood mononuclear cells (PBMC). This latter embodiment is particularly advantageous because it avoids the need to obtain homogeneous populations of cells, which is generally a time-consuming and costly process. The present inventors have also discovered that it is possible to contact such heterogeneous populations with the

- 33 -

overlapping peptides of the invention for short time periods (e.g., as low as 5, 10, 15, 20, 20, 40, 50, 60 min) and infusing the contacted cells directly into a recipient without further culturing of the cells. This further shortens the processing time to permit potentially the harvesting of autologous or syngeneic antigen-presenting cells, treatment of those cells

5   with peptide and infusion of the peptide-contacted cells into a patient in a single sitting or day.

3.2 Delivery of peptide antigen into antigen-presenting cells

The delivery of exogenous peptide antigen to antigen-presenting cells can be enhanced by methods known to practitioners in the art. For example, several different

10   strategies have been developed for delivery of exogenous antigen to the endogenous processing pathway of antigen-presenting cells, especially dendritic cells. These methods include insertion of antigen into pH-sensitive liposomes (Zhou and Huang, 1994, *Immunomethods*, 4:229-235), osmotic lysis of pinosomes after pinocytic uptake of soluble antigen (Moore *et al.*, 1988, *Cell*, 54:777-785), coupling of antigens to potent adjuvants

15   (Aichele *et al.*, 1990, *J. Exp. Med.*, 171: 1815-1820; Gao *et al.*, 1991, *J. Immunol.*, 147: 3268-3273; Schulz *et al.*, 1991, *Proc. Natl. Acad. Sci. USA*, 88: 991-993; Kuzu *et al.*, 1993, *Euro. J. Immunol.*, 23: 1397-1400; and Jondal *et al.*, 1996, *Immunity* 5: 295-302) and apoptotic cell delivery of antigen (Albert *et al.* 1998, *Nature* 392:86-89; Albert *et al.* 1998, *Nature Med.* 4:1321-1324; and in International Publications WO 99/42564 and WO

20   01/85207). Recombinant bacteria (eg. *E. coli*) or transfected host mammalian cells may be pulsed onto dendritic cells (as particulate antigen, or apoptotic bodies respectively) for antigen delivery. Recombinant chimeric virus-like particles (VLPs) have also been used as vehicles for delivery of exogenous heterologous antigen to the MHC class I processing pathway of a dendritic cell line (Bachmann *et al.*, 1996, *Eur. J. Immunol.*, 26(11): 2595-

25   2600).

Alternatively, or in addition, a peptide antigen may be linked to, or otherwise associated with, a cytolysin to enhance the transfer of the peptide into the cytosol of an antigen-presenting cell of the invention for delivery to the MHC class I pathway. Exemplary cytolysins include saponin compounds such as saponin-containing Immune

30   Stimulating Complexes (ISCOMs) (see e.g., Cox and Coulter, 1997, *Vaccine* 15(3): 248-256 and U.S. Patent No. 6,352,697), phospholipases (see, e.g., Camilli *et al.*, 1991, *J. Exp. Med.* 173: 751-754), pore-forming toxins (e.g., an alpha-toxin), natural cytolysins of gram-

- 34 -

positive bacteria, such as listeriolysin O (LLO, e.g., Mengaud *et al.*, 1988, *Infect. Immun.* 56: 766-772 and Portnoy *et al.*, 1992, *Infect. Immun.* 60: 2710-2717), streptolysin O (SLO, e.g., Palmer *et al.*, 1998, *Biochemistry* 37(8): 2378-2383) and perfringolysin O (PFO, e.g., Rossjohn *et al.*, *Cell* 89(5): 685-692). Where the antigen-presenting cell is phagosomal,
5   acid activated cytolysins may be advantageously used. For example, listeriolysin exhibits greater pore-forming ability at mildly acidic pH (the pH conditions within the phagosome), thereby facilitating delivery of vacuole (including phagosome and endosome) contents to the cytoplasm (see, e.g., Portnoy *et al.*, *Infect. Immun.* 1992, 60: 2710-2717).

The amount of peptide antigen to be placed in contact with antigen-presenting
10   cells can be determined empirically by persons of skill in the art. The antigen-presenting cells should be exposed to the peptides for a period of time sufficient for those cells to present the peptides or processed forms thereof for the modulation of T cells. The time and dose of peptides necessary for the cells to optionally process and present the peptides or their processed forms may be determined using pulse-chase protocols in which exposure to
15   peptides is followed by a washout period and exposure to a read-out system e.g., antigen reactive T cells. Once the optimal time and dose necessary for cells to express the peptides or their processed forms on their surface is determined, a protocol may be used to prepare cells and peptides for inducing immunogenic responses. Those of skill in the art will recognise in this regard that the length of time necessary for an antigen-presenting cell to
20   present an antigen on its surface may vary depending on the antigen or form of antigen employed, its dose, and the antigen-presenting cell employed, as well as the conditions under which antigen loading is undertaken. These parameters can be determined by the skilled artisan using routine procedures. Efficiency of priming of the antigen-presenting cells can be determined by assaying T cell cytotoxic activity *in vitro* or using antigen-
25   presenting cells as targets of CTLs. Other methods known to practitioners in the art, which can detect the presence of antigen on the surface of antigen-presenting cells after exposure to one or more of the modified and unmodified antigens, are also contemplated by the presented invention.

Usually, for peptides, about 0.1 to 20 µg/mL to about 1-10 million antigen-
30   presenting cells, is suitable for producing primed antigen-specific antigen-presenting cells. Typically antigen-presenting cells are incubated with peptide for about 1 to 6 hr at a suitably temperature that permits processing and/or presentation of antigen by the antigen-presenting cells (e.g., at about 4, 6, 8, 10, 12, 16° C and desirably at 20, 25, 30, 37° C),

although it is also possible to expose antigen-presenting cells to antigen for the duration of incubation with growth factors and inhibitor. Surprisingly, the present inventors have shown that successful presentation of peptides or their processed forms can be achieved using much shorter periods of incubation (e.g., about 5, 10, 15, 20, 30, 40, 50 minutes)

5 using peptides at a concentration of about 10-20 μg/mL.

4. Lymphocyte embodiments

   The antigen-presenting cells of the invention may be obtained or prepared to contain and/or express one or more antigens by any number of means, such that the antigen(s) or processed form(s) thereof, is (are) presented by those cells for potential

10 modulation of other immune cells, including T lymphocytes and B lymphocytes, and particularly for producing T lymphocytes and B lymphocytes that are primed to respond to a specified antigen or group of antigens. In some embodiments, the subject antigen-presenting cells are useful for producing primed T lymphocytes to an antigen or group of antigens. The efficiency of inducing lymphocytes, especially T lymphocytes, to exhibit an

15 immune response to a specified antigen can be determined by any suitable method including, but not limited to, assaying T lymphocyte cytolytic activity *in vitro* using for example antigen-specific antigen-presenting cells as targets of antigen-specific cytolytic T lymphocytes (CTL); assaying antigen-specific T lymphocyte proliferation (see, *e.g.*, Vollenweider and Groseurth, 1992, *J. Immunol. Meth.* **149**: 133-135), measuring B cell

20 response to the antigen using, for example, ELISPOT assays, and ELISA assays; interrogating cytokine profiles; or measuring delayed-type hypersensitivity (DTH) responses by test of skin reactivity to a specified antigen (see, *e.g.*, Chang *et al.* (1993, *Cancer Res.* **53**: 1043-1050). Other methods known to practitioners in the art, which can detect the presence of antigen on the surface of antigen-presenting cells after exposure to

25 the antigen, are also contemplated by the present invention.

   Accordingly, the present invention also provides antigen-specific B or T lymphocytes, especially T lymphocytes, which respond in an antigen-specific fashion to representation of the antigen. In some embodiments, antigen-specific T lymphocytes are produced by contacting an antigen-presenting cell as defined above with a population of T

30 lymphocytes, which may be obtained from any suitable source such as spleen or tonsil/lymph nodes but is preferably obtained from peripheral blood. The T lymphocytes can be used as crude preparations or as partially purified or substantially purified

preparations, which are suitably obtained using standard techniques as, for example, described in "Immunochemical Techniques, Part G: Separation and Characterization of Lymphoid Cells" (*Meth. in Enzymol.* 108, *Edited by* Di Sabato *et al.*, 1984, Academic Press). This includes rosetting with sheep red blood cells, passage across columns of nylon

5   wool or plastic adherence to deplete adherent cells, immunomagnetic or flow cytometric selection using appropriate monoclonal antibodies is known in the art.

The preparation of T lymphocytes is contacted with the antigen-presenting cells of the invention for an adequate period of time for priming the T lymphocytes to the antigen or antigens presented by those antigen-presenting cells. This period will preferably be at

10 least about 1 day, and up to about 5 days.

In some embodiments, a population of antigen-presenting cells is cultured in the presence of a heterogeneous population of T lymphocytes, which is suitably obtained from peripheral blood, together with a set of peptides of the invention corresponding to an antigen to which an immune response is required. These cells are cultured for a period of

15 time and under conditions sufficient for the peptides, or their processed forms, to be presented by the antigen-presenting cells; and the antigen-presenting cells to prime a subpopulation of the T lymphocytes to respond to the antigen.

5.   Cell based therapy or prophylaxis

The antigen-presenting cells described in Section 3 and the lymphocytes described

20 in Section 4 can be administered to a patient, either by themselves or in combination, for modulating an immune response, especially for priming an immune response to one or more cognate antigens. These cell based compositions are useful, therefore, for treating or preventing a disease or condition as noted above. The cells of the invention can be introduced into a patient by any means (*e.g.*, injection), which produces the desired

25 immune response to an antigen or group of antigens. The cells may be derived from the patient (*i.e.*, autologous cells) or from an individual or individuals who are MHC matched or mismatched (*i.e.*, allogeneic) with the patient. Typically, autologous cells are injected back into the patient from whom the source cells were obtained. The injection site may be subcutaneous, intraperitoneal, intramuscular, intradermal, or intravenous. The cells may be

30 administered to a patient already suffering from a disease or condition or who is predisposed to a disease or condition in sufficient number to treat or prevent or alleviate the symptoms of the disease or condition. The number of cells injected into the patient in

need of the treatment or prophylaxis may vary depending on *inter alia*, the antigen or antigens and size of the individual. This number may range for example between about 10^3 and 10^{11}, and usually between about 10^5 and 10^7 cells (e.g., dendritic cells or T lymphocytes). Single or multiple administrations of the cells can be carried out with cell

5   numbers and pattern being selected by the treating physician. The cells should be administered in a pharmaceutically acceptable carrier, which is non-toxic to the cells and the individual. Such carrier may be the growth medium in which the cells were grown, or any suitable buffering medium such as phosphate buffered saline. The cells may be administered alone or as an adjunct therapy in conjunction with other therapeutics known

10 in the art for the treatment or prevention of unwanted immune responses for example but not limited to glucocorticoids, methotrexate, D-penicillamine, hydroxychloroquine, gold salts, sulfasalazine, TNFα or interleukin-1 inhibitors, and/or other forms of specific immunotherapy.

6. Compositions

15   The overlapping sets of peptides described in Sections 2 and the antigen-primed antigen-presenting cells described in Section 3 or the lymphocytes described in Section 4 (therapeutic/prophylactic agents) can be used singly or together as active ingredients for the treatment or prophylaxis of various conditions associated with the presence of one or more target polypeptide antigens. These therapeutic/prophylactic agents can be

20 administered to a patient either by themselves, or in compositions where they are mixed with a suitable pharmaceutically acceptable carrier and/or diluent, or an adjuvant.

The invention also encompasses a method for stimulating a patient's immune system, and preferably for eliciting a humoral and/or cellular immune response to a polypeptide of interest, by administering to the patient a therapeutic agent or composition

25 as described above. Such stimulation may be utilised for the treatment and/or prophylaxis of a disease or condition including, but not restricted to, a pathogenic infection (e.g., viral, bacterial, fungal, protozoan) or a cancer. Accordingly, the invention contemplates a method for treatment and/or prophylaxis of a disease or condition, comprising administering to a patient in need of such treatment a therapeutically/prophylactically

30 effective amount of a therapeutic agent or composition as broadly described above.

Depending on the specific conditions being treated, therapeutic/prophylactic agents may be formulated and administered systemically or locally. Techniques for

- 38 -

formulation and administration may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, Pa., latest edition. Suitable routes may, for example, include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct

5   intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. For injection, which constitutes one desirable embodiment of the present invention, the therapeutic agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the

10   barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. Intra-muscular and subcutaneous injection is appropriate, for example, for administration of immunogenic compositions, vaccines and DNA vaccines. In certain embodiments of the present invention, the immunogenic compositions are administered intravenously.

15   The therapeutic/prophylactic agents can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated in dosage forms such as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. These carriers may be selected from

20   sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulphate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline, and pyrogen-free water.

       Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to

25   achieve its intended purpose. The dose of agent administered to a patient should be sufficient to effect a beneficial response in the patient over time such as a reduction in the symptoms associated with the condition. The quantity of the therapeutic/prophylactic agent(s) to be administered may depend on the subject to be treated inclusive of the age, sex, weight and general health condition thereof. In this regard, precise amounts of the

30   therapeutic/prophylactic agent(s) for administration will depend on the judgement of the practitioner. In determining the effective amount of the agent to be administered in the treatment or prophylaxis of the condition, the physician may evaluate tissue levels of a

- 39 -

target antigen, and progression of the disease or condition. In any event, those of skill in the art may readily determine suitable dosages of the therapeutic agents of the invention.

5

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain

10

suitable stabilisers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or

15

dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as., for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the

20

cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association one or more therapeutic agents as described above with the carrier which constitutes one or more necessary ingredients. In general, the pharmaceutical compositions of the present invention may be manufactured in

25

a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilising processes.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl

30

pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterise different combinations of active compound doses.

- 40 -

Pharmaceutical which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticiser, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilisers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilisers may be added.

Dosage forms of the therapeutic agents of the invention may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of an agent of the invention may be effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

Therapeutic agents of the invention may be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts may be formed with many acids, including but not limited to hydrochloric, sulphuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms.

For any compound used in the method of the invention, the effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC50 as determined in cell culture (e.g., the concentration of a test agent, which achieves a half-maximal reduction in target antigen). Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the compounds of the invention can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds that exhibit large therapeutic indices are preferred. The data

obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the

5   route of administration utilised. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See for example Fingl *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the active compound(s) which are sufficient to maintain target antigen-reducing

10   effects or effects that ameliorate the disease or condition. Usual patient dosages for systemic administration range from 1-2000 mg/day, commonly from 1-250 mg/day, and typically from 10-150 mg/day. Stated in terms of patient body weight, usual dosages range from 0.02-25 mg/kg/day, commonly from 0.02-3 mg/kg/day, typically from 0.2-1.5 mg/kg/day. Stated in terms of patient body surface areas, usual dosages range from 0.5-

15   1200 mg/m^2/day, commonly from 0.5-150 mg/m^2/day, typically from 5-100 mg/m^2/day.

Alternately, one may administer the agent in a local rather than systemic manner, for example, *via* injection of the compound directly into a tissue, often in a depot or sustained release formulation. Furthermore, one may administer the agent in a targeted drug delivery system, for example, in a liposome coated with tissue-specific antibody. The

20   liposomes will be targeted to and taken up selectively by the tissue.

From the foregoing, it will be appreciated that the agents of the invention may be used as therapeutic or prophylactic immunomodulating compositions or vaccines. Accordingly, the invention extends to the production of immunomodulating compositions containing as active compounds one or more of the therapeutic/prophylactic agents of the

25   invention. Any suitable procedure is contemplated for producing such vaccines. Exemplary procedures include, for example, those described in NEW GENERATION VACCINES (1997, Levine *et al.*, Marcel Dekker, Inc. New York, Basel Hong Kong).

Immunomodulating compositions according to the present invention can contain a physiologically acceptable diluent or excipient such as water, phosphate buffered saline

30   and saline. They may also include an adjuvant as is well known in the art. Suitable adjuvants include, but are not limited to: surface active substances such as hexadecylamine, octadecylamine, octadecyl amino acid esters, lysolecithin,

- 42 -

dimethyldioctadecylammonium bromide, N, N-dicoctadecyl-N', N'bis(2-hydroxyethyl-propanediamine), methoxyhexadecylglycerol, and pluronic polyols; polyamines such as pyran, dextransulfate, poly IC carbopol; peptides such as muramyl dipeptide and derivatives, dimethylglycine, tuftsin; oil emulsions; and mineral gels such as aluminum
5   phosphate, aluminum hydroxide or alum; lymphokines, QuilA and immune stimulating complexes (ISCOMS).

The antigen-primed antigen-presenting cells of the invention and antigen-specific T lymphocytes generated with these antigen-presenting cells, as described *infra*, can be used as active compounds in immunomodulating compositions for prophylactic or
10 therapeutic applications. The primed cells, which are preferably mature dendritic cells, can be injected by any method that elicits an immune response into a syngeneic animal or human. Preferably, antigen-presenting cells are injected back into the same animal or human from whom the source tissue/cells was obtained. The injection site may be subcutaneous, intraperitoneal, intramuscular, intradermal, or intravenous. The number of
15 antigen-primed antigen-presenting cells reinjected back into the animal or human in need of treatment may vary depending on *inter alia*, the antigen and size of the individual. This number may range for example between about 10^4 and 10^8, and more preferably between about 10^6 and 10^7 antigen-primed antigen-presenting cells (e.g., dendritic cells). The antigen-presenting cells should be administered in a pharmaceutically acceptable carrier,
20 which is non-toxic to the cells and the individual. Such carrier may be the growth medium in which the antigen-presenting cells were grown, or any suitable buffering medium such as phosphate buffered saline.

In one embodiment, the antigen-primed antigen-presenting cells of the invention could also be used for generating large numbers of $CD8^+$ or CD4+ CTL, for adoptive
25 transfer to immunosuppressed individuals who are unable to mount normal immune responses. For example, antigen-specific $CD8^+$ CTL can be adoptively transferred for therapeutic purposes in individuals afflicted with HIV infection (Koup *et al.*, 1991, *J. Exp. Med.*, 174: 1593-1600; Carmichael *et al.*, 1993, *J. Exp. Med.*, 177: 249-256; and Johnson *et al.*, 1992, *J. Exp. Med.*, 175: 961-971), malaria (Hill *et al.*, 1992, *Nature*, 360: 434-439)
30 and malignant tumours such as melanoma (Van der Brogen *et al.*, 1991, *Science*, 254: 1643-1647; and Young and Steinman, 1990, *J. Exp. Med.*, 171: 1315-1332).

In another embodiment, the immunomodulating composition of the invention is suitable for treatment or prophylaxis of a cancer. Cancers which could be suitably treated

- 43 -

in accordance with the practices of this invention include cancers associated with a viral infection such as cervical cancer (e.g., papillomavirus infection) and Burkitt's lymphoma (e.g., Epstein Barr virus infection). Other virus associated cancers include, but are not restricted to, HTLV1 associated leukemia, Non Hodgkins lymphoma (EBV), anal cancer,

5   skin cancer (HPV), hepatocellular carcinoma (HBV) and Kaposis sarcoma (HHV8). Alternatively, the cancer may be a non-virally associated cancer such as but not limited to melanoma, lung cancer, breast cancer, prostate cancer, colon cancer, pancreatic cancer, stomach cancer, bladder cancer, kidney cancer, post transplant lymphoproliferative disease (PTLD), Hodgkin's Lymphoma and the like.

10   In yet another embodiment, the immunomodulating composition is suitable for treatment or prophylaxis of a viral, bacterial or protozoan infection. Viral infections contemplated by the present invention include, but are not restricted to, infections caused by HIV, Hepatitis, Influenza, Japanese encephalitis virus, Epstein-Barr virus and respiratory syncytial virus. Bacterial infections include, but are not restricted to, those

15   caused by *Neisseria* species, *Meningococcal* species, *Haemophilus* species *Salmonella* species, *Streptococcal* species, *Legionella* species and *Mycobacterium* species. Protozoan infections encompassed by the invention include, but are not restricted to, those caused by *Plasmodium* species (e.g., malaria), *Schistosoma* species (e.g., schistosomiasis), *Leishmania* species, *Trypanosoma* species, *Toxoplasma* species and *Giardia* species.

20   *7.   Methods for assessing immunomodulation*

        The effectiveness of the immunisation may be assessed using any suitable technique. An individual's capacity to respond to foreign or disease-specific antigens (e.g., viral antigens and cancer antigens) may be determined by assessing whether those cells primed to attack such antigens are increased in number, activity, and ability to detect and

25   destroy those antigens. Strength of immune response is measured by standard tests including: direct measurement of peripheral blood lymphocytes by means known to the art; natural killer cell cytotoxicity assays (see, e.g., Provinciali M. *et al* (1992, *J. Immunol. Meth.* 155: 19-24), cell proliferation assays (see, e.g., Vollenweider, I. and Groseurth, P. J. (1992, *J. Immunol. Meth.* 149: 133-135), immunoassays of immune cells and subsets (see,

30   e.g., Loeffler, D. A., *et al.* (1992, *Cytom.* 13: 169-174); Rivoltini, L., *et al.* (1992, *Can. Immunol. Immunother.* 34: 241-251); or skin tests for cell-mediated immunity (see, e.g., Chang, A. E. et al (1993, *Cancer Res.* 53: 1043-1050). Alternatively, the efficacy of the

- 44 -

immunisation may be monitored using one or more techniques including, but not limited to, HLA class I tetramer staining - of both fresh and stimulated PBMCs (see for example Allen *et al.*, *supra*), proliferation assays (Allen *et al.*, *supra*), ELISPOT assays and intracellular cytokine staining (Allen *et al.*, *supra*), ELISA Assays - for linear B cell

5   responses; and Western blots of cell sample expressing the synthetic polynucleotides. Particularly relevant will be the cytokine profile of T cells activated by antigen, and more particularly the production and secretion of IFN γ, IL-2, IL4, IL5, IL-10, TGFβ and TNF α.

The cytotoxic activity of T lymphocytes, and in particular the ability of cytotoxic

10 T lymphocytes to be induced by antigen-presenting cells, may be assessed by any suitable technique known to those of skill in the art. For example, a sample comprising T lymphocytes to be assayed for cytotoxic activity is obtained and the T lymphocytes are then exposed to antigen-primed antigen-presenting cells, which have been caused to present antigen. After an appropriate period of time, which may be determined by

15 assessing the cytotoxic activity of a control population of T lymphocytes which are known to be capable of being induced to become cytotoxic cells, the T lymphocytes to be assessed are tested for cytotoxic activity in a standard cytotoxic assay.

The method of assessing CTL activity is particularly useful for evaluating an individual's capacity to generate a cytotoxic response against cells expressing tumour or

20 viral antigens. Accordingly, this method is useful for evaluating an individual's ability to mount an immune response to a cancer or virus. For example, CTL lysis assays may be employed using stimulated splenocytes or peripheral blood mononuclear cells (PBMC) on peptide coated or recombinant virus infected cells using ^{51}Cr labelled target cells. Such assays can be performed using for example primate, mouse or human cells (Allen *et al.*,

25 2000, *J. Immunol.* 164(9): 4968-4978 also Woodberry *et al.*, *infra*).

In order that the invention may be readily understood and put into practical effect, particular preferred embodiments will now be described by way of the following non-limiting experimental example.

- 45 -

EXAMPLE

MATERIALS AND METHODS

Animals

      18 Male juvenile, colony-bred pigtailed macaques (*Macaca nemestrina*, aged 2-4
5   years) were studied. All animals were housed under PC3 biosafety conditions by trained
animal technicians at the CSIRO Australian Animal Health Laboratory, Geelong. Prior to
all procedures, animals were anaesthetised with ketamine (10 mg/kg, intramuscularly).
Health and weight were routinely monitored. All conditions and protocols were approved
by the CSIRO animal health and the University of Melbourne animal ethics committees.

10   *Pre-immunisations*

      To evaluate whether the OPAL method could boost T cell responses in animals
with pre-primed responses. T cell responses were induced in macaques by administering 2
DNA vaccines expressing HIV or SIV structural genes followed by a FPV boost vaccine
expressing similar HIV or SIV genes as previously described (16). DNA vaccines in saline
15   were administered twice intramuscularly (0.5 ml to each anterior quadracep) at a dose of
1mg/dose. FPV boosts were delivered intramuscularly a dose of 5×10^7 pfu.

Isolation of plasma and peripheral blood mononuclear cells (PBMC) from whole blood

      Blood was collected in 9ml Na+ Heparin and 3 mL EDTA vacutainers from the
femoral vein of each animal on study weeks prior to and after vaccination and SHIV
20   challenge. Plasma samples were removed following centrifugation (800×g, room
temperature, RT, 8 min; Beckman Coulter) and stored in 3×1.5-mL tubes at -70° C. Plasma
collected in EDTA-anticoagulated blood was used for RNA extraction. Media (RPMI-1640
supplemented with penicillin, streptomycin and glutamine; Invitrogen) equal to the volume
of plasma collected was added to the blood and mixed prior to PBMC isolation on Ficoll-
25   Paque, used according to the manufacturer's instructions (Amersham Pharmacia). PBMC
were washed twice (500×g, 10° C, 6min) and resuspended in 1 mL media for counting
(Beckman Coulter Counter®) in preparation of immunological assays.

Overlapping peptides

      15-mer peptides (>80% purity) overlapping by 11 amino acids spanning the entire
30   gag (125 peptides), pol (260 peptides) and nef (21 peptides) of SIV$_{mac239}$ and env (211

peptides) protein of SHIV$_{SF162P3}$ (NIH AIDS Research and Reference Reagent depository) (Figure 1-4) were pooled for each protein by solubilising each 1mg peptide aliquot in 10-40 μL of DMSO to final concentrations: SIV$_{mac239}$ gag (670μg/ml or 730μg/ml); pol (304 μg/ml), and; nef (4.762 mg/mL), and; SHIV$_{SF162P3}$ env (330 μg/mL), stored at -70°C until use.

SIV antigens for in vitro analyses

Whole inactivated SIV (WI SIV) and its control (supernatant from Hut78-CLE cell-line used to culture the WI SIV) (AIDS Vaccine Program, National Cancer Institute, MD) were stored at -70°C until use.

In vivo cytotoxic T lymphocyte killing

At weeks 10, 15 and 20 following the initial vaccination, PBMC from the macaques were isolated from 40-50 mL blood, as described above. 25 mL sterile injectable saline was infused into the animals immediately after blood sampling to prevent hypovolemia. PBMC were resuspended in PBS and divided into 3 or 4 equal volumes, 0.5ml. Cells were pulsed with SIVgag, pol, nef or SHIVenv peptide pools (10 μg/mL) or DMSO (volume of equal to the volume of SIVgag), in PBS for 90 min at 37° C, or on ice, with regular mixing. To subsequently track each peptide-pulsed cell population by flow cytometry, each peptide/DMSO-pulsed population was then labelled with a concentration of CFSE or SNARF (Molecular Probes). 5mM CFSE stock in DMSO at-20° C was thawed and diluted in PBS. Neat SNARF stock was dissolved in 83 μLDMSO to make 1mM and diluted in PBS. Table 1 shows the final concentrations of each dye. Cells were mixed thoroughly and stained for 10 min in a 37° C waterbath, followed by one wash in RF5 then PBS (500×g, 10°C, 6min). All peptide/DMSO-pulsed cells for each animal were pooled in 1.5ml saline for re-infusion into the femoral vein. 3ml blood was sampled from the opposite femoral vein at 5 min, and at 4 and 16hr following infusion. Red blood cells were lysed with 10ml FACS Lysing Solution (BD Biosciences), incubated for 10min at room temp. Cells were pelleted and washed twice with PBS (800×g, RT, 7min), and fixed with 1-2ml 2% paraformaldehyde (Figure 5).

To determine whether cell populations were being selectively killed, 10^6 events gated live lymphocytes were collected by flow cytometry (FACSort Calibre, BD). CFSE and SNARF fluorescence were detected by FL1 and FL2 channels, respectively. For

- 47 -

analysis, killing was expressed as the percentage of target versus control peptide-pulsed cell clearance. In the event of acquiring unequal labeled populations by flow cytometry at 5 minutes post-OPAL infusion, the degree of killing was subsequently scaled with respect to the initial population ratios obtained at 5 minutes. PBMC were also analysed prior to, and one week following, OPAL-infusion by IFNγ ELISpot and ICS to detect whether immune responses were enhanced.

SHIV challenge of macaques

To assess the efficacy to the vaccines, each macaque was inoculated intrarectally with SHIV$_{mn229}$ (5×10^4 TCID$_{50}$/mL on CD8-depleted *M.nemestrina* PBMC) in 0.5 mL doses over 2 days (total 10^5 TCID$_{50}$/mL) 18 weeks after the initial immunisation, as previously described (32).

Quantification of viral SHIV RNA by reverse-transcriptase real-time PCR

RNA extraction: To detect SHIV RNA in macaques following SHIV challenge, total RNA was initially extracted from stored plasma samples from anti-coagulated blood collected in EDTA with QIAamp® Viral RNA commercial kit (Qiagen) as previously described (32). Briefly, plasma samples were centrifuged (500×g, RT, 10min) to remove cells (preventing DNA contamination). 140 µL plasma RNA coupled to Carrier RNA in Buffer AVL and 96-100% ethanol was centrifuged and bound to a filter membrane. 60 µL RNA was eluted with Buffer AW1 and AW2 through a spin column. All reagents except ethanol supplied by kit.

Reverse-transcriptase PCR: 10 µL RNA was then reverse transcribed into cDNA, in duplicate, with the reaction mixture (20 µL): 2.9 µL RNAse/DNAse-free water (Promega); 3 µL 10× Taqman buffer A (Applied Biosystems); 6 µL MgCl$_2$ (25nM) (Applied Biosystems); 1.5 µL Randon Hexamers (diluted 1/2; Applised Biosystems); 6µl dNTPs (2.5nM; Promega); 1.5 µL; Promega); 0.5 µL Rnasin (40U/mL; Promega); 0.1 µL MMLV-RT superscript (200U/mL; Invitrogen), for one thermal cycle: 25° C (15min) → 42° C (40min) → 75° C (5min) (GeneAmp PCR System 9700, Applied Biosystems). A third test per sample was set up to assess the presence of SHIV DNA contamination, using the same reaction mix excluding MMLV-RT superscript. SIV RNA standards (33) were serially diluted and reverse-transcribed in duplicate (limit of detection, 1500 copies/mL).

- 48 -

Real-time PCR: cDNA was amplified with reaction mixture (20 µl): 141µl RNAse/DNAse-free water (Promega); 2µL 10× PCR buffer II (Applied Biosystems); 1 µL MgCl₂ (Applied Biosystems); 1 µL SL03 SIVgag (20pmol/µL); 1µL SL04 SIVgag (20 pmol/□L); 0.3 µL SL07 molecular beacon 0.5µL Tag Gold (Applied Biosystems) as previously described (33). Reaction temperature was initially raised and held at 95 °C for 10 min to activate Tag Gold enzyme, followed by 45 thermal cycles: 95° C (15 sec) → 55° C (30 sec) → 72 °C (30 sec). Real-time analysis was performed on amplicon detection at 55° C (30 sec) stages by Sequence Detector software v1.6.3 (Applied Biosystems).

CD4+ T cell counts

To assess the depletion of CD4+ T cells following SHIV challenge, 200 µL whole blood was incubated with 5 µL PE-conjugated anti-human CD3, 5 µL FITC-conjugated anti-human CD4, 5 µL PerCP-conjugated anti-human CD8 (clone SP34; L200, and; Leu-2a, respectively; BD Pharmingen) monoclonal antibodies for 20 min in dark, RT. Red blood cells were lysed with 2 ml FACS Lysing Solution (BD Biosciences) and fixed as described in method 2.8. 50,000 total events were collected by 3-colour FACScan Calibre® and CD4+ and CD8+ T cell counts expressed as the percentage of gated lymphocytes.

RESULTS

In vivo cytotoxic T-lymphocyte killing

The standard measure of virus-specific CTL effector is measured *via* the release of a radioisotope ^{51}Cr from target cells, an assay that is tedious and poorly sensitive. By pulsing dye-labelled autologous macaque PBMC with large pools of SIV and SHIV overlapping peptides (OPAL) and infusing the cells back into the same animal, the inventors were able to kinetically show SHIV-specific killing in blood sampled at various time-points following the infusion of OPAL by flow cytometry.

Two weeks after full immunisation (week 10), three of four immunised animals displayed moderate to large (11.4-76%) killing of gag-pulsed PBMC by 16 hours post-OPAL infusion, whereas control-immunised monkeys displayed <7% gag-specific killing. One immunised animal, monkey H20, demonstrated vigorous gag-specific killing (27.3%) as early as 4 hours post-infusion (Figure 6). These data were consistent with T cell responses induced by the vaccines as analysed by IFNγ ELISpot and ICS (data not shown),

- 49 -

indicating the usefulness of OPAL to measure effective CTL effector responses primed by the DNA and FPV vaccines.

Shortly (2 weeks) after SHIV intrarectal challenge all four immunised animals exhibited large degrees of gag-specific killing (65-98.3%) 16 hours post-OPAL infusion,
5 and two of four (monkeys H20 and H21) further demonstrated >99% pol-specific killing (Figure 7). In comparison with control-immunised animals, monkey E20 displayed <6% killing of both gag- and pol-pulsed PBMC whereas monkey E22 showed >90% and 31.9% of gag- and pol-pulsed PBMC, respectively. Interestingly, the animals that displayed moderate to high degrees of pol-specific killing (monkeys H20, H21 and E22) were also
10 the only animals that had previously received 2 doses of infused pol-pulsed PBMC (weeks 10 and 15), whereas monkeys B00, H8 and E20 received pol-pulsed PBMC only once prior. This observation suggests that the infusion of OPAL may have: (a) boosted pol-specific T cell responses primed by the vaccines that were weakly or not detected by IFNγ ELISpot and ICS (data not shown), and; (b) induced pol-specific immunity in naïve
15 animals evident post-SHIV challenge.

Analysis of the immunogenicity induced by infusing peptide-pulsed autologous cells.

It seemed plausible that if *in vivo* CTL killing could be efficiently measured by OPAL infusion, this method may be able to either prime a new or boost an existing immune response. IFNγ ELISpot and ICS assays were therefore performed prior to- and
20 one week following each OPAL infusion assay to analyse whether there would be an increase in T cell immunogenicity previously primed by the vaccines or by the OPAL infusion method itself (Figure 8).

Following the first OPAL infusion performed at week 10, a 3- to 16-fold increase in IFNγ-secreting cells to SIV gag peptide pool was detected in monkeys H20 and H21,
25 measuring up to 430 spot-forming cells (Figure 9). Monkey H8 measured a 54% increase to 215 spot-forming cells, whereas no increase was measured in control-immunised animals. Analysis of monkeys B00 (post-OPAL infusion) and E20 (pre-OPAL infusion) for all antigens analysed were excluded due to developmental problems of the assay. Of the four animals that received pol-pulsing at week 10, monkeys H20, H21 and E22,
30 displayed increased pol responses by up to 140 spot-forming cells post-OPAL infusion, whereas no significant ELISpot responses were detected in monkey E20. No nef-specific T cell was in all animals apparent before or after OPAL-infusion. These results suggest a

- 50 -

boosting effect in T cell immunogenicity following gag- and pol-peptide pulsing in the animals previously primed for SIVgag/pol responses, and furthermore indicate priming for SIVpol in a naïve animal (monkey E22).

At week 15, 8 weeks following full immunisation, a second OPAL infusion assay was performed in the six animals. ELISpot analyses revealed increased responses to gag peptide pool by up to 500 spot-forming cells from approximately 50 or less spot-forming cells prior to OPAL infusion in the four animals pre-immunised with DNA and FPV vaccines. In control-immunised animals, no gag-specific T cells were measured before or after the assay (Figure 10). In comparison, a slight increase in pol-specific responses (up to 40 spot-forming cells) from baseline was measured in only a few animals. Large increased responses to WI SIV were measured in all pre-immunised animals (up to 450 spot-forming cells), whereas control-immunised animals displayed modest or no increases (up to 50 spot-forming cells). All responses to SIV nef and SHIV env were minimal or undetected in all animals prior to and after OPAL infusion.

Following SHIV intrarectal challenge, all animals except monkey E20 displayed increased gag responses measuring between 50-600 spot-forming cells. Similar responses were observed for WI SIV but to levels up to 200 spot-forming cells, whereas pol responses above 50 spot-forming cells were only evident in monkey H20.

The immunogenicity of OPAL infusion was further verified by comparison to animals that received the same immunisation regimen but did not receive OPAL infusion (Figure 11). No rise in SIV gag, pol or WI SIV-specific T cells were detected in groups 1 (control-immunised) and 2 (2×DNA/FPV-immunised) from weeks 9 to 11 and 15 to 18. Responses from weeks 20 to 21 increased slightly the groups, attributable to responses enhanced by SHIV challenge at week 18.

Outcome of SHIV~mn229~ intrarectal challenge

The highly pathogenic SHIV$_{mn229}$ challenge stock was inoculated intrarectally into all macaques 10 weeks after full immunisation at a dose of 10^5 TCID$_{50}$. Plasma SHIV RNA and CD4+ T cell counts were followed in all control-and 2×DNA/FPV-immunised animals (Figure 12).

Control-immunised monkeys E20 and E22 exhibited peak viral loads of 7.8±0.7 log$_{10}$ copies/ml at 2 weeks following challenge. The peak viral load of monkey E20 may

have occurred between week 1 and 2, however, set-point levels of both monkeys (measured 5 to 11 weeks post challenge) remained high at 5.9 ± 0.3 $\log_{10}$ copies/mL. Conversely at week 2, CD4+ T cell counts dropped dramatically to $1.6\pm1.1\%$ of total lymphocytes, and set-point levels were steady at $0.3\pm0.2\%$. Monkeys that received the
5   same immunisations but no OPAL infusions (group 1) performed only marginally worse than monkeys E20 and E22 in terms of peak and set-point viral loads (8.2 ± 0.1 $\log_{10}$ copies/ml and 6.2 ± 0.3 $\log_{10}$ copies/ml), as well as CD4+ counts (set-point $0.5\pm0.3\%$).

Based on the enhanced pol-specific killing that may have been attributed to 2 separate OPAL infusions, the SHIV viral loads and CD4+ T cell counts of monkeys H20
10   and H21 were compared to monkeys B00 and H8 that received only 1 dose of pol-OPAL infusions. Peak viral load of monkeys H20 and H21 (receiving 2 pol-OPAL infusions) was at least 10-fold lower than monkeys B00 and H8 (5.9 ± 1.3 vs. 7.1 ± 0.4 $\log_{10}$ copies/mL, $P=0.08$), and set-point viral load showed a trend towards being lower (4.1 ± 0.9 vs. 5.4 ± 0.7 $\log_{10}$ copies/ml, $P=0.08$, student's t test). Incidentally, set-point CD4+ T cell count for
15   monkeys H20 and H21 was significantly greater than monkeys B00 and H8 ($18.9\pm6.1\%$ vs. 8.4%, $P=0.02$). Although statistically insignificant in comparison with group 2 animals who received the same immunisations but no OPAL infusions ($P=0.12$), monkeys H20 and H21 that received multiple pol-OPAL infusions displayed a trend towards the retainment of CD4+ T cells although viral loads were relatively similar, indicative of viral challenge
20   protection. Set-point CD4+ T cell count and viral load of group 2 were $13.0\pm3.7\%$ and 4.8 ± 0.2 $\log_{10}$ copies/ml, respectively.

In comparison to control-immunised monkeys E20 and E22, both set-point viral load and CD4+ T cell count of monkeys H20 and H21 were significantly different ($P=0.01$, $P=0.00$). The set-point viral load of monkeys B00 and H8, on the other hand, was
25   not significantly lower than monkeys E20 and E22 ($P=0.37$) despite significant set-point levels of CD4+ T cells ($P=0.01$). Note that monkey H20 had completely cleared plasma viral RNA from week 5 and onwards and retained CD4+ T cells at normal levels.

DISCUSSION

The vital role for HIV-1-specific CD4+ T-helper (Th) and CD8+ CTL responses
30   in controlling HIV-1 replication is the focus of many current vaccine concepts. The infusion of autologous PBMC pulsed with large overlapping sets of SHIV 15mer peptides (OPAL) was surprisingly immunogenic in its ability to boost SHIV-specific immune

responses as analysed by IFNγ ELISpot and ICS assays. This finding forms the potential basis of a novel vaccine or immunotherapeutic strategy as described herein.

The evidence for this immunogenicity was five-fold: (a) Increases in SIV gag-specific IFNγ ELISpot responses were observed one week after each of the 3 SIV gag

5   OPAL infusions (week 10, 15, and 20) in all vaccinated monkeys. In contrast, at week 10 and 15, SIVgag responses in equivalently immunised animals (group 2) not receiving the OPAl infusion significantly declined. (b) Increases in SIV pol-specific IFNγ ELISpot responses were observed in immunised animals one week following the SIV pol infusion at week 10 and 20. Interestingly this was observed in only the two monkeys H20 and H21

10   that received multiple SIV pol OPAL infusions prior to SHIV challenge (weeks 10 and 15) and not in animals receiving SIV pol peptide pulsed cells at week 15. This is of particular interest since the pol-specific T cell responses to the DNA and FPV vaccines alone were modest or undetectable by ELISpot and ICS. (c) High levels of SIV pol-specific *in vivo* killing were also seen in the two monkeys that received 2 prior infusions of SIV pol OPAL

15   infusions. (d) This immunogenicity data was further confirmed by high levels of SIV pol-specific IFNγ intracellular cytokine responses in the two immunised animals receiving the multiple SIV pol OPAL infusions. (e) There was a trend towards greater protection from SHIV challenge in animals receiving multiple OPAL infusions. Together, these results suggest that pulsing autologous PBMC *ex vivo* with pools of overlapping peptides is an

20   effective method for boosting immune responses.

There is a body of data ascertaining the use of pulsing autologous or syngeneic cells with defined peptide epitopes or whole antigen for the induction (or 'cross-priming') of immune responses (22, 23, 27, 34, 35). The use of specialised antigen presenting cells such as monocyte-derived dendritic cells pulsed with, for example, single tumour antigens

25   or whole inactivated SIV has also been studied extensively as an immunotherapeutic tool (36-39). However, to the inventors' knowledge this is the first report of utilising large peptide pools spanning an entire protein (125 SIV gag 15mers or 263 SIV pol 15mers) and the use of whole PBMC cultured for short periods *ex vivo*, as a method of boosting immune responses.

30   In one control-immunised animals, monkey E22, which received multiple infusions of SIV pol (and SIV gag) pulsed cells, a modest induction of SIV gag and SIV pol-specific IFNγ ELISpot responses was detected. This animal subsequently had high

levels of SIV gag- and pol-specific killing analysed at week 20, presumably from the boosting effect of the SHIV challenge. The efficiency of priming an immune response by OPAL infusion therefore seems feasible.

5   Further modifications to the OPAL technique, such as the enrichment for APC and/or dendritic cells (DC) (40), would potentially enhance the immunogenicity of OPAL infusion as a therapeutic vaccine since DC cultured from PBMC of HIV-infected patients (41, 42) and SIV-infected animals (40) can elicit potent T-cell responses. Alternatively, the prospect of using whole blood rather than PBMC fractions as a means of delivering OPAL will certainly benefit a clinical setting, particularly for HIV-infected persons. Furthermore,

10   a smaller whole blood sample may not require as high a concentration of peptide since $1 \mu g/mL$ is effective *in vitro* for whole blood analysis by ICS. It is also conceivable that direct intravenous infection of pooled peptides would mimic the immunogenicity of the OPAL effect. The use of consensus HIV-1 clade peptide sets of gag and pol offers the broad epitopic breadth desired of an effective therapeutic vaccine for humans. The

15   immunogenicity of antigens that regulate viral replication, such as rev, tat, vpu, vif and vpr, which are poorly immunogenic by current vaccine approaches, should also be improved using this strategy.

The disclosure of every patent, patent application, and publication cited herein is hereby incorporated herein by reference in its entirety.

20   The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The

25   invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

DATED this 10 June, 2003

THE UNIVERSITY OF MELBOURNE

30   By their Patent Attorneys

DAVIES COLLISON CAVE

BIBLIOGRAPHY

1. Piot, P., et al., *The global impact of HIV/AIDS*. Nature, 2001. 410(6831): p. 968-73.

2. UNAIDS, *Global estimates of HIV/AIDS epidemic as of end 2002*. 2003, UNAIDS.

3. Borrow, P., et al., *Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection.* J Virol, 1994. 68(9): p. 6103-10.

4. Koup, R.A., et al., *Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome.* J Virol, 1994. 68(7): p. 4650-5.

5. Musey, L., et al., *Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection.* N Engl J Med, 1997. 337(18): p. 1267-74.

6. Ogg, G.S., et al., *Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA.* Science, 1998. 279(5359): p. 2103-6.

7. Carmichael, A., et al., *Quantitative analysis of the human immunodeficiency virus type 1 (HIV- 1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection: differential CTL responses to HIV-1 and Epstein- Barr virus in late disease.* J Exp Med, 1993. 177(2): p. 249-56.

8. Rinaldo, C., et al., *High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1-infected long-term nonprogressors.* J Virol, 1995. 69(9): p. 5838-42.

9. Hay, C.M., et al., *Lack of viral escape and defective in vivo activation of human immunodeficiency virus type 1-specific cytotoxic T lymphocytes in rapidly progressive infection.* J Virol, 1999. 73(7): p. 5509-19.

10. Johnson, P.R., et al., *Inactivated whole SIV vaccine in macaques: evaluation of protective efficacy against challenge with cell-free virus or infected cells.* AIDS Res Hum Retroviruses, 1992. 8(8): p. 1501-5.

- 55 -

11.   Cranage, M.P., et al., *Studies on the specificity of the vaccine effect elicited by inactivated simian immunodeficiency virus.* AIDS Res Hum Retroviruses, 1993. **9**(1): p. 13-22.

12.   Luke, W., et al., *Simian immunodeficiency virus (SIV) gp130 oligomers protect rhesus macaques (Macaca mulatta) against the infection with SIVmac32H grown on T-cells or derived ex vivo.* Virology, 1996. **216**(2): p. 444-50.

13.   Mooij, P., et al., *A clinically relevant HIV-1 subunit vaccine protects rhesus macaques from in vivo passaged simian-human immunodeficiency virus infection.* Aids, 1998. **12**(5): p. F15-22.

14.   VaxGen, I., *VaxGen Announces Initial Results of its Phase III AIDS Vaccine Trial.* 2003, PR Newswire.

15.   Kent, S.J., et al., *Vaccination with attenuated simian immunodeficiency virus by DNA inoculation.* J Virol, 2001. **75**(23): p. 11930-4.

16.   Kent, S.J., et al., *Enhanced T-cell immunogenicity and protective efficacy of a human immunodeficiency virus type 1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus.* J Virol, 1998. **72**(12): p. 10180-8.

17.   Santra, S., et al., *Prior vaccination increases the epitopic breadth of the cytotoxic T- lymphocyte response that evolves in rhesus monkeys following a simian- human immunodeficiency virus infection.* J Virol, 2002. **76**(12): p. 6376-81.

18.   Estcourt, M.J., et al., *Prime-boost immunization generates a high frequency, high-avidity CD8(+) cytotoxic T lymphocyte population.* Int Immunol, 2002. **14**(1): p. 31-7.

19.   Marzo, A.L., et al., *Tumor-specific CD4+ T cells have a major "post-licensing" role in CTL mediated anti-tumor immunity.* J Immunol, 2000. **165**(11): p. 6047-55.

20.   Nelson, D.J., et al., *Tumor progression despite efficient tumor antigen cross-presentation and effective "arming" of tumor antigen-specific CTL.* J Immunol, 2001. **166**(9): p. 5557-66.

21. Ritchie, D.S., et al., *Dendritic cell elimination as an assay of cytotoxic T lymphocyte activity in vivo*. J Immunol Methods, 2000. **246**(1-2): p. 109-17.

22. Carbone, F.R. and M.J. Bevan, *Induction of ovalbumin-specific cytotoxic T cells by in vivo peptide immunization*. J Exp Med, 1989. **169**(3): p. 603-12.

23. Carbone, F.R., et al., *Induction of cytotoxic T lymphocytes by primary in vitro stimulation with peptides*. J Exp Med, 1988. **167**(6): p. 1767-79.

24. Kast, W.M., et al., *Protection against lethal Sendai virus infection by in vivo priming of virus-specific cytotoxic T lymphocytes with a free synthetic peptide*. Proc Natl Acad Sci U S A, 1991. **88**(6): p. 2283-7.

25. Schulz, M., R.M. Zinkernagel, and H. Hengartner, *Peptide-induced antiviral protection by cytotoxic T cells*. Proc Natl Acad Sci U S A, 1991. **88**(3): p. 991-3.

26. Feltkamp, M.C., et al., *Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells*. Eur J Immunol, 1993. **23**(9): p. 2242-9.

27. Celluzzi, C.M., et al., *Peptide-pulsed dendritic cells induce antigen-specific CTL-mediated protective tumor immunity*. J Exp Med, 1996. **183**(1): p. 283-7.

28. Kearney, E.R., et al., *Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo*. Immunity, 1994. **1**(4): p. 327-39.

29. Aichele, P., et al., *T cell priming versus T cell tolerance induced by synthetic peptides*. J Exp Med, 1995. **182**(1): p. 261-6.

30. Toes, R.E., et al., *Enhanced tumor outgrowth after peptide vaccination. Functional deletion of tumor-specific CTL induced by peptide vaccination can lead to the inability to reject tumors*. J Immunol, 1996. **156**(10): p. 3911-8.

31. Toes, R.E., et al., *Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction*. Proc Natl Acad Sci U S A, 1996. **93**(15): p. 7855-60.

32. Dale, C.J., et al., *Chimeric human papilloma virus-simian/human immunodeficiency virus virus-like-particle vaccines: immunogenicity and protective efficacy in macaques*. Virology, 2002. **301**(1): p. 176-87.

33. Jin, X., et al., *Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques.* J Exp Med, 1999. **189**(6): p. 991-8.

34. Carbone, F.R. and M.J. Bevan, *Class I-restricted processing and presentation of exogenous cell- associated antigen in vivo.* J Exp Med, 1990. **171**(2): p. 377-87.

35. Larsson, M., et al., *Efficiency of cross presentation of vaccinia virus-derived antigens by human dendritic cells.* Eur J Immunol, 2001. **31**(12): p. 3432-42.

36. Chen, Q., et al., *A direct comparison of cytolytic T-lymphocyte responses to Melan-A peptides in vitro: differential immunogenicity of Melan-A27-35 and Melan-A26-35.* Melanoma Res, 2000. **10**(1): p. 16-25.

37. Lu, W., et al., *Therapeutic dendritic-cell vaccine for simian AIDS.* Nat Med, 2003. **9**(1): p. 27-32.

38. Pospisilova, D., et al., *Generation of functional dendritic cells for potential use in the treatment of acute lymphoblastic leukemia.* Cancer Immunol Immunother, 2002. **51**(2): p. 72-8.

39. Melief, C.J., et al., *Effective therapeutic anticancer vaccines based on precision guiding of cytolytic T lymphocytes.* Immunol Rev, 2002. **188**(1): p. 177-82.

40. Mehlhop, E., et al., *Enhanced in vitro stimulation of rhesus macaque dendritic cells for activation of SIV-specific T cell responses.* J Immunol Methods, 2002. **260**(1-2): p. 219-34.

41. Sapp, M., et al., *Dendritic cells generated from blood monocytes of HIV-1 patients are not infected and act as competent antigen presenting cells eliciting potent T-cell responses.* Immunol Lett, 1999. **66**(1-3): p. 121-8.

42. Chougnet, C., et al., *Normal immune function of monocyte-derived dendritic cells from HIV-infected individuals: implications for immunotherapy.* J Immunol, 1999. **163**(3): p. 1666-73.

FIGURE 1

TLLIQMANPDCKLVIAKGLGVNPTLEEMLTACQVGGPEQKARLMAEALKEALAPVPIPFAAAQQRGPRKPIKCHNCGREGHSARQCRAPRRQGCWXCGKQGHVMAKCPDRQAGFLGLGFWGKKQRNFPMAQVHQGLMPTAPPEDPAVDLLKNYMQLGKQQREKQRESREKP
(81) TLLIQMANPDCKLVL
QMANPDCKLVIKGLG
PDCKLVIKGLGVNPT
LVIKGLGVNPTLEEK
GLGVNPTLEEMLTAC
NPTLEEMLTACQVG
EEMLTACQVGGPEQ
TACQVGGPEQKARL
GVGGPEQKARLMAEA
(90) PGQKARLMAEALKEA
ARLMAEALKEALAPV
AEALKEALAPVPIPF
KEALAPVPIPFAAAQ
APVPIPFAAAQQRGP
IPFAAAQQRGPRKPI
AAQQRGPRKPIKCHN
RGPRKPIKCHNCGKE
XPIKCNRCGKEGHSA
CHNCGREGHSARQCR
(100) GKEGHSARQCRAPRR
(101) HSARQCRAPRRQGCW
QCRAPRRQGCWKCGK
PRRQGCWKCGKWGHV
GCWKCGKWGHV
CGKWDHVMAKC
DHVMAKCPDRQ
AKCPDRQAGFL
DRQAGFLGLGP
GFLGLGFWGKKPRNF
(110) LGPWGKKPRNFPMAQ
GKKPRNFPMAQVHQG
RNFPMAQVHQGLMPT
MAQVHQGLMPTAPPE
HQGLMPTAPPEDPAV
MPTAPPEDPAVDLLK
PPEDPAVDLLKNYMQ
PAVDLLKNYMQLGKQ
LLKNYMQLGKQQREK
YMQLGKQQREKQRES
(120) GKQQREKQRESREKP

REKQRESREKPYKEVTEDLHLHLNSLFGGDQ
(121) REKQRESREKPYKEV
RESREKPYKEVTEDL
EKPYKEVTEDLHLHN
KEVTEDLHLHNSLFG
(125) EDLHLHLNSLFGGDQ

FIGURE 1 contd

VLELMERGTLCKAMQSPKKTGRLEMKHNNGPCYGQMPRQVQGPPRPHSMGKEAPQFPHGSSASGADANCSPRGPSCGSAKELHAVGQAAERKAERKQREALQGGDRGPAAPQFSLMRRPVVTAHIEGQPVEVLLDTGADDSIVTGIELGPHYTPKIVGGIGGPINTKE
(1) VLELMERGTLCKAMQ

NERGTLCKAMQSPKK
TLCKAMQSPKKTGRL
AMQSPKKTGRLEMKH
PKKTGRLEMKHNNGPC
GRLEMKHNNGPCYGQM
MKHNNGPCYGQM
GPCYGQMPRQTGGFP
GQMPRQTGGFP
(10) RQTGGFPRPHSMGKE
GPPRPHSMGKE
PHSMGKEAPQFPHGS
GKEAPQFPHGSSASG
PQFPHGSSASGADAN
HGSSASGADANCSPR
ASGADANCSPRGPSC
DANCSPRGPSCGSAK
SPRGPSCGSAKELHA
PSCGSAKELHAVGQA
(20) SAKELHAVGQAAERK

(21) LHAVGQAAERKAERK
GQAAERKAERKQREA
ERKAERKQREALQGG
ERKQREALQGGDRGP
REALQGGDRGPAAPQ
QGGDRGPAAPQFSLM
RGPAAPQFSLMRRPV
APQFSLMRRPVVTAH
SLMRRPVVTAHIEGQ
(30) RPVVTAHIEGQPVEV
TAHIEGQPVEVLLDT
EGQPVEVLLDTGADD
VEVLLDTGADDSIVT
LDTGADDSIVTGIEL
ADDSIVTGIELGPHY
IVTGIELGPHYTPKI
IELGPHYTPKIVGGI
PHYTPKIVGGIGGPI
PKIVGGIGGPINTKE
(40) GGIGGPINTKSYKNV

GPINTKSYKNVEIEVLGKRIKGTIVMGDTPINIFGRLLITALGHSLANFPIAKVEPVKVALKPGKDGPRKLKQWPLSKEKVALRBICEKMKKDGQLSEAPPTNPYNTPTFAIKKDKGKKRHLIDFRELMRVTQDFTEVQLGIPHPAGLAKKRRITVLDIGDAYFSIPLDEE
(41) GPINTKSYKNVEIEV

INTKSYKNVEIEVLG
KSYKNVEIEVLGKRI
KNVEIEVLGKRIKGT
IEVLGKRIKGTIVMG
GKRIKGTIVMGDTPI
KGTIVMGDTPINIFG
IVMGDTPINIFGRLL
MGDTPINIFGRLL
TPINIFGRLLITALG
IFGRLLITALGHSLN
(50) NLITALGHSLANFPIA
ALGHSLANFPIAKVEP
SLANFPIAKVEPVKVA
PIAKVEPVKVALKEG
VEPVKVALKPGKDGP
KVALKPGKDGPRLKQ
KPGKDGPRLKQWPLS
DGPRLKQWPLSKEKI
LKQWPLSKEKIVALR
PLSKEKIVALREICE
(60) EKIVALREICEKMEK

(61) ALREICEKMKKDGQL
ICEKMKKDGQLSEAP
MKKDGQLSEAPPTNP
GQLSEAPPTNPYNTP
SEAPPTNPYNTPTFAI
TNPYNTPTFAIKKD
NTPTFAIKKDKGKKRH
FAIKKDKGKKRHLI
KKDKGKKRHLIDFRE
(70) KGKKRHLIDFRELNRV
LIDFRELNRVTQDF
FRELNRVTQDFTEVQ
NRVTQDFTEVQLGIP
QDFTEVQLGIPHPAG
EVQLGIPHPAGLAKKR
GIPHPAGLAKKRIT
PAGLAKKRRITVLDI
AKKRRITVLDIGDAY
RITVLDIGDAYFSIP
(80) LDIGDAYFSIPLDEE

FIGURE 2

(81) DAYFSIPLDEEFRQYTAFTLPSVNRAEPGKRVIYKVLPQGNKGSPAIFQYTMRHVLEPFRKANPDVTLVQYTMDDILLIASDRTDLEHDRVVLQSKELLNSIGFSTPEEKFQDDPPFQMMGYELNPTKWKLQXIELPQRETWTVNDIQKLVGVLNWAAQIYPGIKTGHL

(90) LPGGNKGSPAIFQYT

(100) IASDRTDLEHDRVVL

(101) RTDLEHDRVVLQSKE

(110) MNGYELNPTKWKLQK

(120) QIYPGIKTHLCRLGI

(121) GIKTKHLCRLIRGKMTLZEEVQWTEHARAYEENKIILSQEDEGCYYQEGKPLRATVIKSQDNQWSYKIHQEDKILKVGKFAKIKTHTNGVRLLAHVIQKGKEAIVIWGQVPKFHLPVEKDVHEQWMTDYWQVTHIPERDFISTPPLVRLVFNLVKDPIEGEGTYYTDG

(130) ILSQEDEGCYYQEGK

(140) KVGKFAKIKTHTNG

(150) HLPVEKDVHEQWMTD

(160) VKDPIEGEGTYYTDG

FIGURE 2 contd

(161) IEGBETYYTDGSCHKQSKEGRAGYITDEGRDKVKVLEQTTNQQASLEAFLNALTDSGPKANIIVDSQYVWGIITGCPTESESRLVNQIIEEMIKKSEIYVAWVPAHKGIGGNQEIDHLVSQGIRQVLFLEKIEPAQEEHDKYHSNVKELVFKFGLPRIVARQIVDTCDKCH

(181) ESRLVNQIIEEMIKK

VNQIIEEMIKKSEIY

IEEMIKKSEIYVAWV

IKKSEIYVAWVPAHK

EIYVAWVPAHKGIGG

AWVPAHKGIGGNQEI

AHKGIGGNQEIDHLV

IGGNQEIDHLVSQGI

QEIDHLVSQGIRQVL

(190) HLVSQGIRQVLFLEK

QGIRQVLFLEKIEPA

QVLFLEKIEPAQEEH

LEKIEPAQEEHDKYH

EEHDKYHSNVKELVF

KYHSNVKELVFKFGL

BVKELVFKFGLPRIV

LVFKFGLPRIVARQI

FGLPRIVARQIVDTC

(200) RIVARQIVDTCDKCH

IEGBETYYTDGSCHKQSKE

ETYYTDGSCHKQSKE

TDGSCHKQSKEGRAG

CHKQSKEGRAGYITD

SKEGRAGYITDRGRD

RAGYITDRGRDKVKV

ITDRGRDKVKVLEQT

GRDKVKVLEQTTNQQ

(170) EQTTNQQASLEAFLN

NQQASLEAFLNALTD

ELEAFLNALTDSGPK

FLNALTDSGPKANII

LTDSGPKANIIVDSQ

GPKANIIVDSQYVWG

NIIVDSQYVWGIITG

DSQYVWGIITGCPTE

VWGIITGCPTESESR

ITGCPTESESRLVNQ

(180) PTESESRLVNQIIEE

(201) RQIVDTCDKCHQKGEAIHGQANSDLGTWQDCTHLEGKIIVAVHVASGFIEABVIPQBTGRQTALFLLKLQAGRHPITHLHTDNGANFASQEVKMVAWRAGIEHTFGVPYNPQSQGVVEANVHHLKNQIDRIREQANSVETIVLMAVHCMNFKRGGIGDMTPAERLINMI

(221) HTDNGANFASQEVKM

GANFASQEVKMVAWR

ASQEVKMVAWRAGIE

VKMVAWRAGIEHTFG

AWRAGIEHTFGVPYN

GIEHTFGVPYNPQSQ

TFGVPYNPQSQGVVE

PYNPQSQGVVEANVH

QSQGVVEANVHHLKN

(230) VVEANVHHLKNQIDR

VHHLKNQIDRIREQ

LKNQIDRIREQANSV

IDRIREQANSVETIV

REQANSVETIVLMAV

NSVETIVLMAVHCMN

TIVLMAVHCMNFKGR

MAVHCMNFKRGGIG

CMNFKRGGIGDMTP

KRGGIGDMTPAERL

(240) GIGDMTPAERLINMI

RQIVDTCDKCHQKGE

DTCDKCHQKGEAIHG

CDKCHQKGEAIHG

KCHQKGEAIHGQANS

KGEAIHGQANSDLGT

IHGQANSDLGTWQKD

ANSDLGTWQDCTHL

LGTWQDCTHLEGKI

QDDCTHLEGKIIVA

(210) GKIIVAVHVASGFI

THLEGKIIVAVHVA

IVAVHVASGFIEABV

TVAVHVASGFIEAEV

HVASGFIEABVIPQE

GFIEABVIPQETGRQ

ABVIPQETGRQTALF

PQETGRQTALFLLKL

GRQTALFLLKLQAGRM

ALFLLKLQAGRMPITH

LKLQAGRMPITHLHTD

GRMPITHLHTDNGAN

(220) ITHLHTDNGANFASQ

FIGURE 2 contd

MTPAERLINMIITEQEIQFQQSRNSKFKNFRVYYREGRDQLMKGEGELLMKGEGAVILKVGTDIKVVPRRKAKIIKDYGGGGKEVDSSSHWEDTGEAREVA
(241) MTPAERLINMIITEQ   (261) GKEVDSSSHWEDTGE
ERLINMIITEQEIQF   DSSSHWEDTGEAREV
NMIITEQEIQFQQSK   (263) HWEDTGEAREVA
TEQEIQFQQSRNSKF
IQFQQSRNSKFKNFR
QSRNSKFKNFRVYYR
SKFKNFRVYYREGRD
NFRVYYREGRDQLMK
YYREGRDQLMKGPGE
(250) GRDQLMKGPGELLMK
LMKGPGELLMKGEGA
PGELLMKGEGAVILK
LMKGEGAVILKVGTD
EGAVILKVGTDIKVV
ILKVGTDIKVVPRRK
GTDIKVVPRRKAKII
KVVPRRKAKIIKDYG
RRKAKIIKDYGGGGKE
KIIKDYGGGGKEVDSS
(260) DYGGGGKEVDSSSHWE

FIGURE 2 contd

```
     MGGAISHRSRPSGDLRQRLLRARGETYGRLLGEVEDGYSQSPGGLDKGLLSSLSCEGQKYNQGQYTWTPWRNPAEEREKLAYRKQNMDDIDE
(21)                                                                                    AYRKQNMDDIDE
(1)  MGGAISHRSRPSGD
        ISMRSRPSGDLRQR
           RSRPSGDLRQRLLRA
              SGDLRQRLLRARGET
                 RQRLLRARGETYGRL
                    LRARGETYGRLLGEV
                       GETYGRLLGEVEDGY
                          GRLLGEVEDGYSQSP
                             GEVEDGYSQSPGGLD
(10)                            DGYSQSPGGLDKGLS
                                   QSPGGLDKGLSSLSC
                                      GLDKGLSSLSCEGQK
                                         GLSSLSCEGQKYNQG
                                            LSCEGQKYNQGQYWN
                                               GQKYNQGQYWTPWR
                                                  NQGQYWTPWRNPAE
                                                     YWTPWRNPAEEREK
                                                        PWRNPAEEREKLAYR
                                                           PAEEREKLAYRKQNM
(20)                                                          REKLAYRKQNMDDID
```

FIGURE 3

FIGURE 4

IIGDIRQAHCNISGSHWNNTLKQIVTKLQAQFENKTIVFKQSSGGDPEIVMHSFNCGGEFFYCNSTQLFNSTWNNTIGPNNTNGTITLPCRIKQIINRRQEVGKAWAPPIRGQIRCSSNITGLLLTRQGGREVGNTTEIFRPGGGDMRDNWRSELYKYKVVKIEPLGV
(81) IIGDIRQAHCNISGS
IRQAHCNISGEKNAN
HCNISGEKNNTLKQ
SGEKNNTLKQIVTK
NNNTLKQIVTKLQAQ
LKQIVTKLQAQFENK
VTKLQAQFENKTIVF
LQAQFENKTIVFKQS*
FENKTIVFKQSSGGD
(90) TIVFKQSSGGDPEIV
KQSSGGDPEIVMHSF
GGDPEIVMHSFNCGG
EIVMHSFNCGGEFFY
HSFNCGGEFFYCNST
CGGEFFYCNSTQLFN
FFYCNSTQLFNSTWN
NSTQLFNSTWNNTIG
LFNSTWNNTIGPNNT
TWNNTIGPNNTNGTI
(100) TIGPNNTNGTITLPC

NMTNGTITLPCRIKQ
(101) NMTNGTITLPCRIKQ
GNTTLPCRIKQIINR
LPCRIKQIINRRQEV
IKQIINRRQEVGKAW
INRRQEVGKAWTAPP
RQEVGKAWTAPPIRG*
GKAWTAPPIRGQIRC
YAPPIRGQIRCSSNI
IRGQIRCSSNITGLL
(110) IRCSSNITGLLLTRD
SSNITGLLLTRDGGR
GLLLTRDGGREVGNT
TRDGGREVGNTTEIP
GREVGNTTEIFRPGG
GNTTEIFRPGGGDNR
EIFRPGGGDNRDNWR
PGGGDMRDNWRSELY
DMRDNWRSELYKYKV
NWRSELYKYKVVKIE
(120) ELYKYKVVKIEPLGV

YKVVKIEPLGVAPTKAKRRVVQREKRAVTLGANVFLGFLGAAGSTMGAASLTLTVQARQLLSGIVQQQNNLLRAIEAQQRLLQLTVWGIKQLQARVLAVERYLKDQQLLGIWGCSGKLICTTAVPWNASRSNKSLDQIWNNMTWMEWEREIGNYTNLIYTLIEESQNQQE
(121) YKVVKIEPLGVAPTK
KIEPLGVAPTKAKRR
LGVAPTKAKRRVVQR
PTKAKRRVVQREKRA
KRRVVQREKRAVTLG
VQREKRAVTLGANVFL
KRAVTLGANVFLGFLG
TLGANVFLGFLGAAGS
VFLGFLGAAGSTMGA
(130) FLGAAGSTMGAASLT
AGSTMGAASLTLTVQ
MGAASLTLTVQARQL
SLTLTVQARQLLSGI
TVQARQLLSGIVQQQ
RQLLSGIVQQQNNLL
SGIVQQQNNLLRAIE
VQQQNNLLRAIEAQQ
NNLLRAIEAQQRLLQ
RAIEAQQRLLQLTVW
(140) AQQRLLQLTVWGIKQ

LLQLTVWGIKQLQAR
(141) LLQLTVWGIKQLQAR
TVWGIKQLQARVLAV
IKQLQARVLAVERYL
LQARVLAVERYLKDQ*
VLAVERYLKDQQLLG
ERYLKDQQLLGIWGC
KDQQLLGIWGCSGKL
LLGIWGCSGKLICTT
WGCSGKLICTTAVPW
(150) GKLICTTAVPWNASW
ICTTAVPWNASWSNKS
AVPWNASWSNKSLDQ
ASWSNKSLDQIWNNM
NKSLDQIWNNMTWME
DQIWNNMTWMEWERE
NNMTWMEWEREIGNY
WMEWEREIGNYTNLI
EREIGNYTNLIYTLI
GNYTNLIYTLIEESQ
(160) ELIYTLIEESQNQQE

FIGURE 4 contd

(161) TLIEBSQQQEKNEQBLLELDKRAASLNRVLDISKWLMYIKIFIMIVGGLVGLRIVFTVLSIVNRVRQGYSPLSPQTRFPAPRGLDRPEGIEBGGERDRDRSRPLVHGLLALLIWDDLRSLCLFSYHRLRDLILLAARIVELLGRRGWEALKYWGNLLQYWIQELKNSAVS
TLIEBSQQQEKNEQ
ESQMQQEKNEQELLE
NQQEKNEQBLLELDK*
KNEQBLLELDKRASL
ELLELDKRASLNRWL
LDKRASLNRVLDISK
ASLNRVLDISKWLMY
NRVLDISKWLMYIKIF
ISKWLMYIKIFIMIV
(170) LMYIKIFIMIVGGLV
KIFIMIVGGLVGLRI
MIVGGLVGLRIVFTV
GLVGLRIVFTVLSIV
LRIVFTVLSIVNRVR
FTVLSIVNRVRQGYS
SIVNRVRQGYSPLSP
RVRQGYSPLSPQTRF
GYSPLSPQTRFPAPR
LSPQTRFPAPRGLDR
(180) TRFPAPRGLDRPEGI
APRGLDRPEGIEBGG
LDRPEGIEBGGERD
EGIEBGGERDRDRS
EBGGERDRDRSRPLV
ERDRDRSRPLVHGLL
DRSRPLVHGLLALLIW
PLVHGLLALLIWDDLR
GLLALLIWDDLRSLCL
LLIWDDLRSLCLFSYH
(190) DLRSLCLFSYHRLRD
LCLFSYHRLRDLILI
SYHRLRDLILIAARI
LRDLILIAARIVELL
ILIAARIVELLGRRG
ARIVELLGRRGWEAL
ELLGRRGWEALKYWG
. RRGWEALKYWGNLLO
EALKYWGNLLQYWIQ
YWGNLLQYWIQELKN
(200) LLQYWIQELKNSAVS

(201) WIQELKNSAVSLFGAIALAVAEGTDRIIEVAQRIGRAFLHIPRRIRQGLERTLL
WIQELKNSAVSLFGA
LKNSAVSLFGAIAIA
AVSLFGAIAIAVAEG
FGAIAIAVAEGTDRI
AIAVAEGTDRIIEVA
AEGTDRIIEVAQRIG
DRIIEVAQRIGRAFL
EVAQRIGRAFLHIPR
(210) AFLHIPRRIROGLER
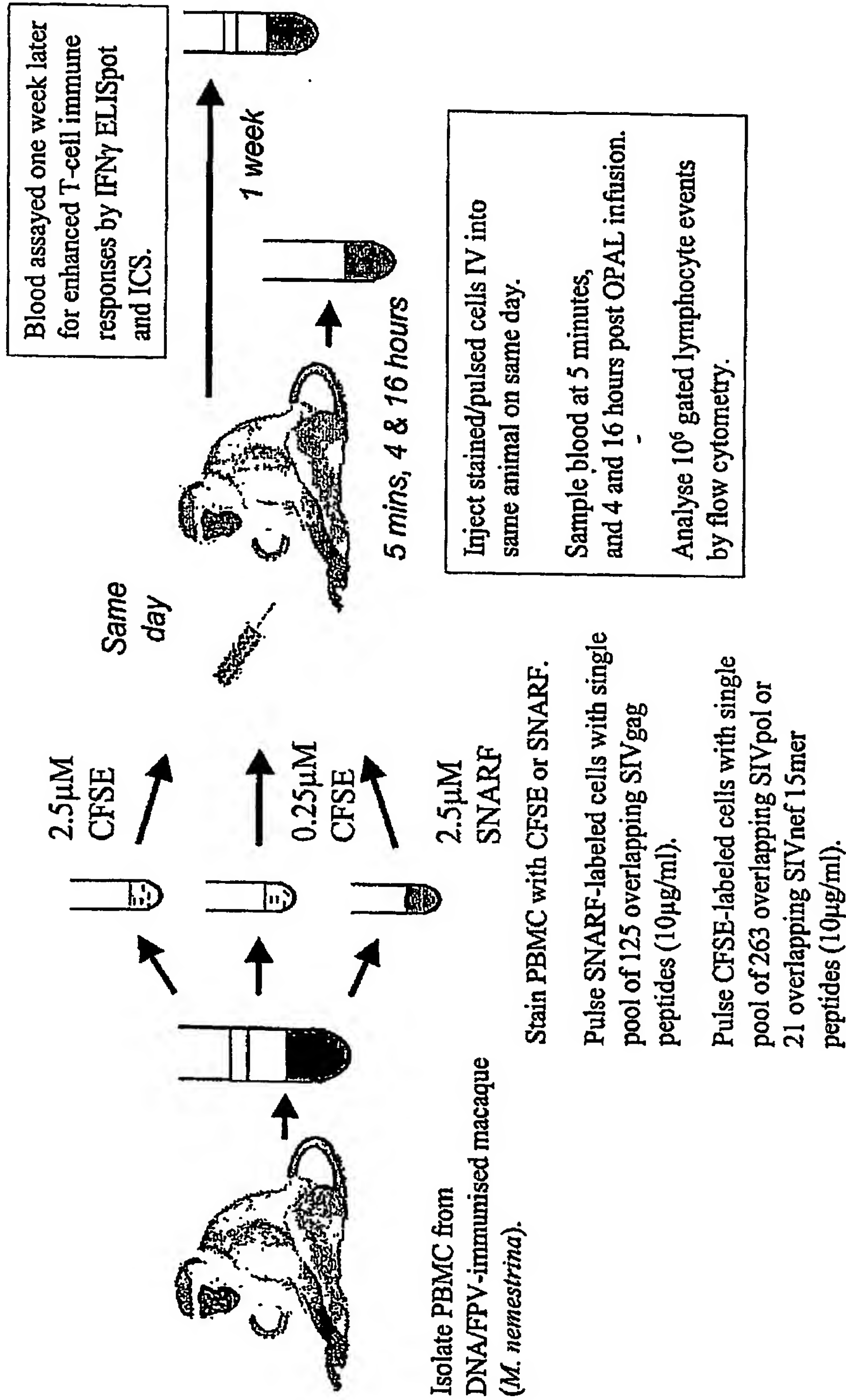RIGRAFLHIPRRIRQ
(211) IPRRIRQGLERTLL

FIGURE 4 contd

FIGURE 5
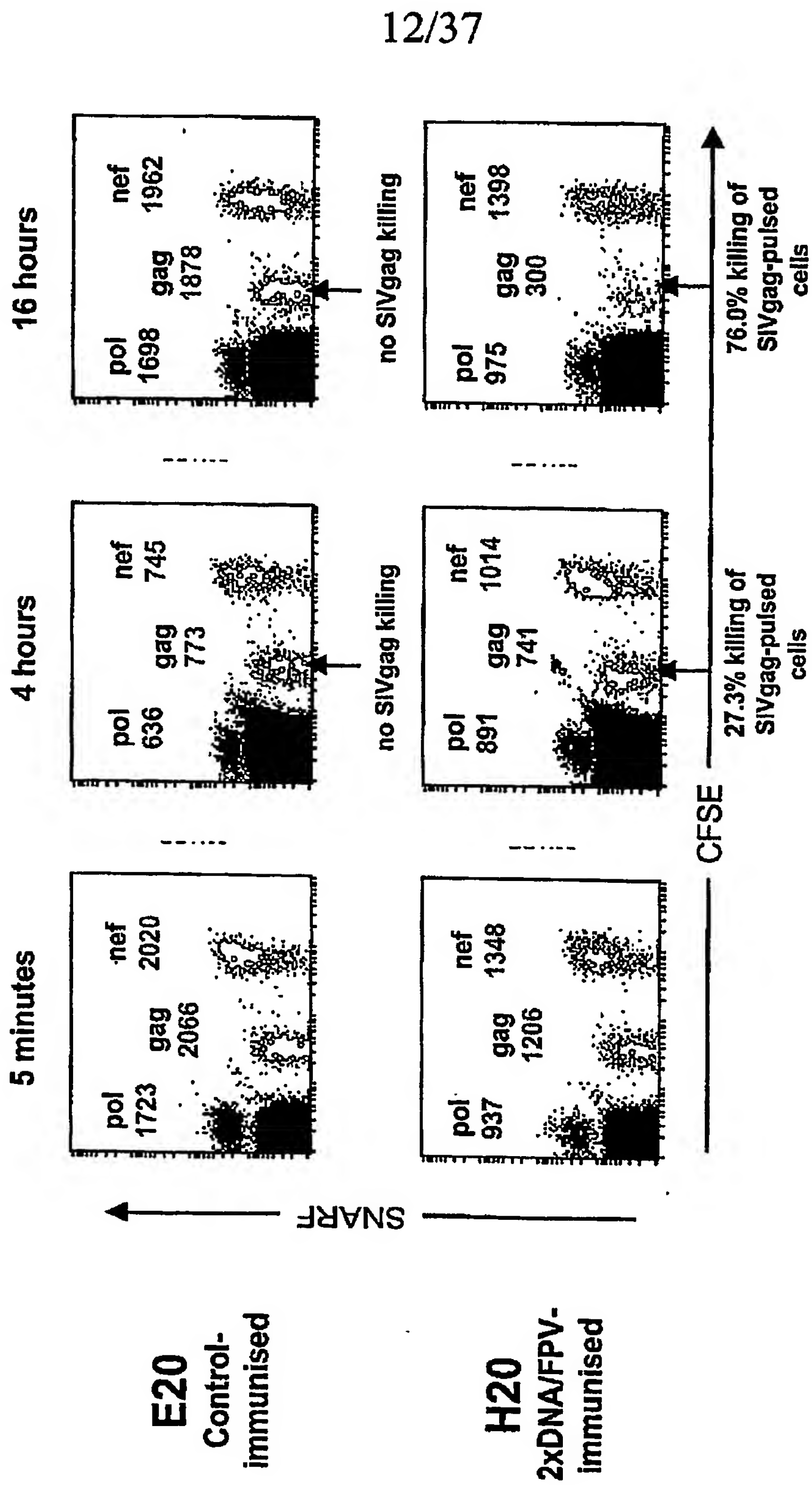
FIGURE 6
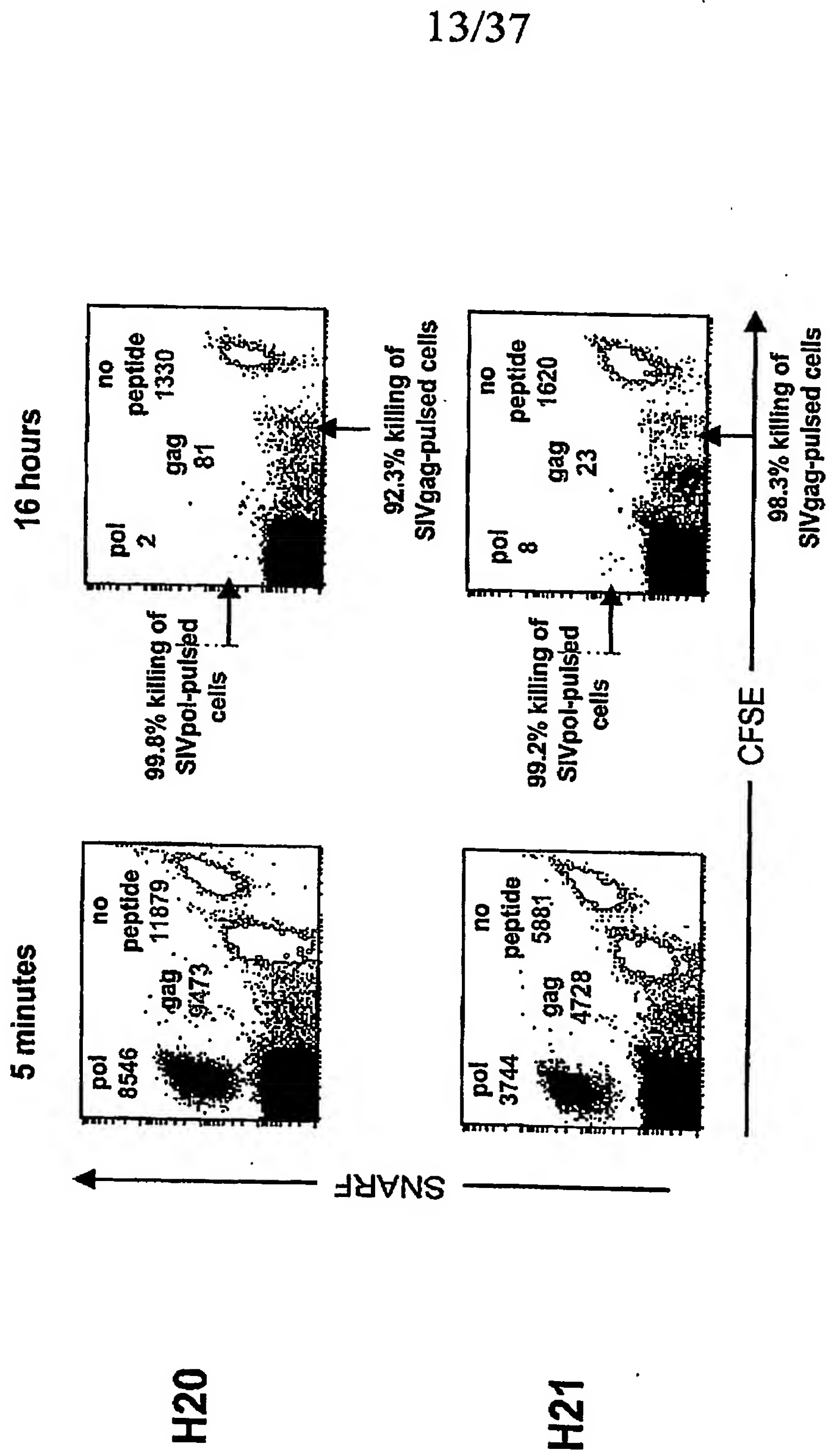
FIGURE 7

E22 - control-immunised animal
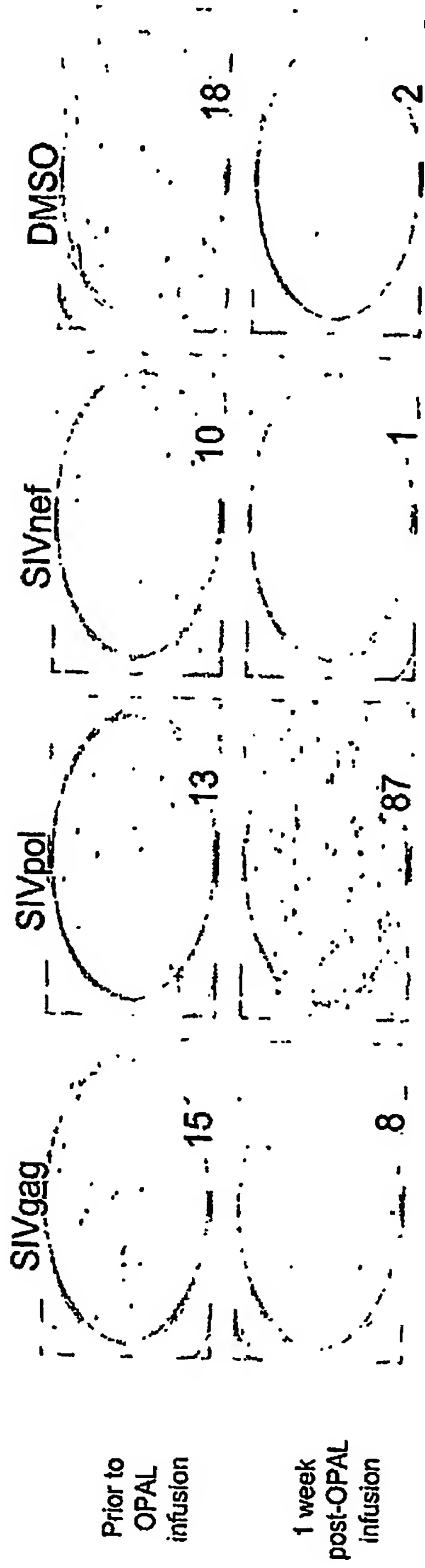
H20 - 2xDNA/FPV-immunised animal
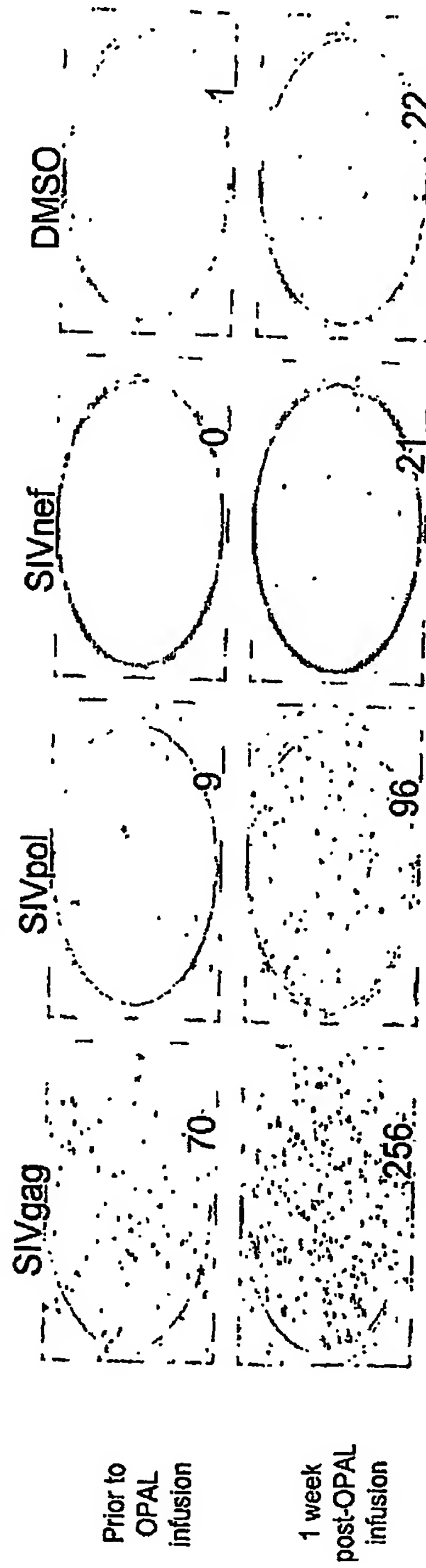
FIGURE 8
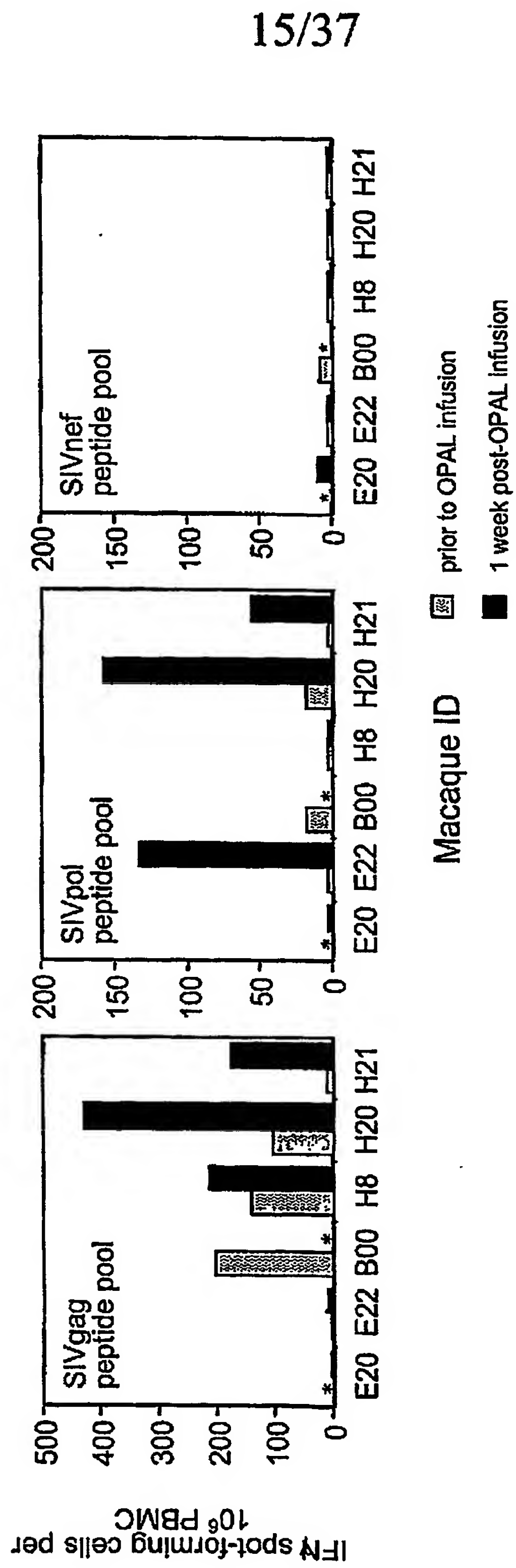
FIGURE 9
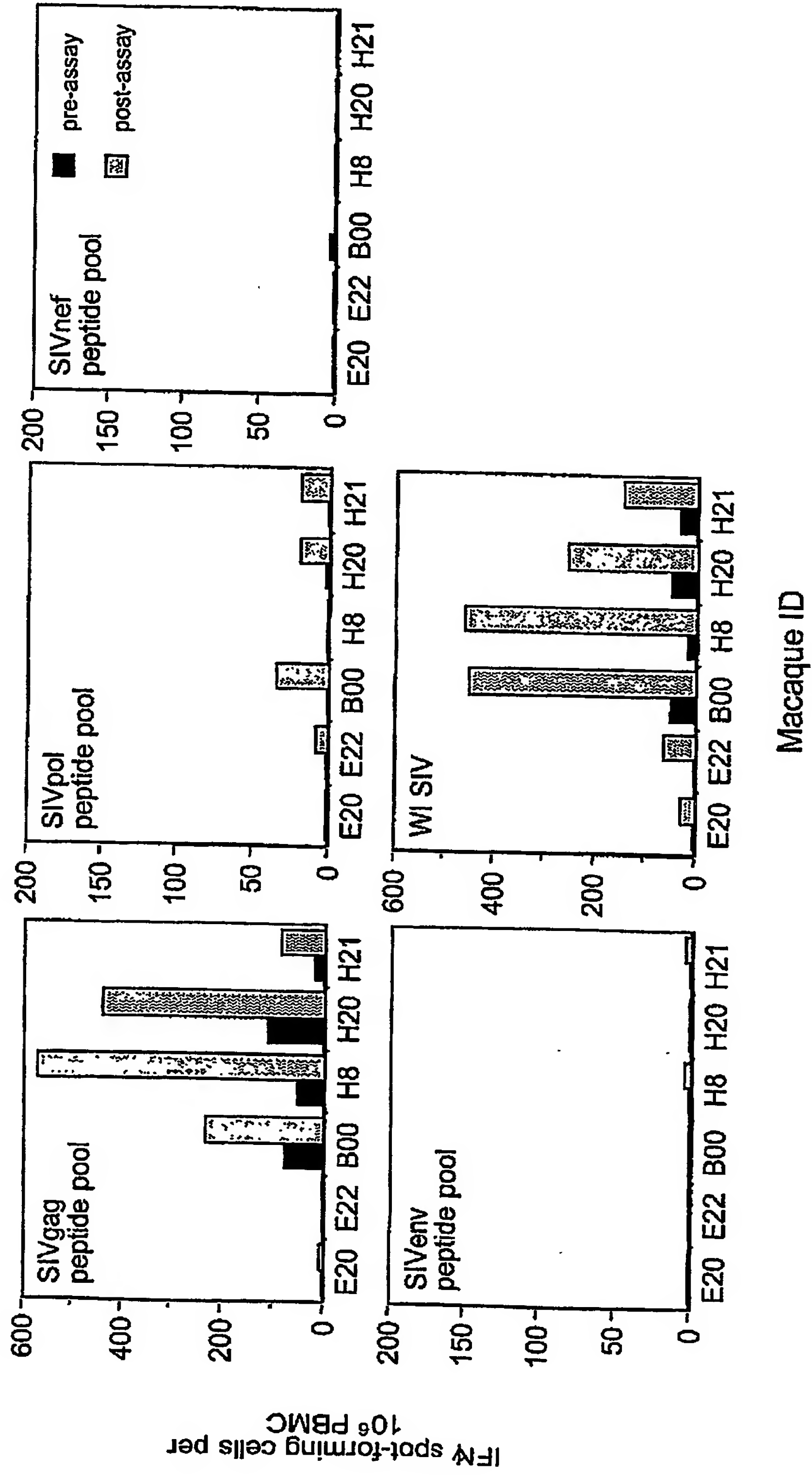
FIGURE 10

FIGURE 11

FIGURE 12

FIGURE 13

ETLLVGNAPDCKTILKALGPAATLEENTACQGVGCPGHKARVLAEAMSQVTNSATINHQRGRFRNQRKTVKCPNCGKEGHIAKNCRAPRKKGCWKCGKEGHQMKDCTERQANPLGKIWPSHKGRPGNFLQSRPEPTAPPEESFRFGEETTTPSQKQEPIDKELYPLA

(81) ETLLVGNAPDCKTI
VGNAPDCKTILKALGPAA
NPDCKTILKALGPAA
KTILKALGPAATLEE
KALGPAATLEENTA
PAATLEENTACQGV
LEENTACQGVGCPG
MTACQGVGCPGHKAR
QCVGCPGHKARVLAE
(90) GPGHKARVLAEAMSQ
KARVLAEAMSQVTNS
LAEAMSQVTNSATIN
MSQVTNSATINNQRG
TNSATINNQRGRFRN
TINNQRGRFRNQRK.
QRGRFRNQRKTVKC
FRNQRKTVKCPNCG
RKTVKCPNCGKEGH
VKCPNCGKEGHIAKN
(100) NCGKEGHIAKNCRAP

(101) EGHIAKNCRAPRKKG
AKNCRAPRKKGCWKC
RAPRKKGCWKCGKEG
KKGCWKCGKEGHQMK
RKCGKEGHQMKDCTE
KEGHQMKDCTERQAN
QMKDCTERQANPLGK
CTERQANPLGKIWPS
QANPLGKIWPSHKGR
(110) LGKIWPSHKGRPGNF
WPSHKGRPGNFLQSR
KGRPGNFLQSRPEPT
GNFLQSRPEPTAPPE
QSRPEPTAPPEESFR
PEPTAPPEESFRFGE
EPTAPPEESFRFGE
PFEESFRFGEETTI
SFRFGEETTTPSQK
GEETTTPSQKQEPI
TTTPSQKQEPIDKEL
(120) SQKQEPIDKELYPLA

EPIDKELYPLASLRSLFGNDPSSQ
(121) EPIDKELYPLASLRS
KELYPLASLRSLFGN
PLASLRSLFGNDPSS
(124) LRSLFGNDPSSQ

FIGURE 13 contd

MGGKWSKRSVVGWPTVRERMRRAEPAADGVGAVSRDLEKHGAITSSNTAANNADCAWLEAQEEEEVGFPVRPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIYSQKRQDILDLWVHTQGYFPDQNYTPGQIRYPLTFGWCFKLVPVEPEKVEEANEGENNSLLHPMSLH

(1) MGGKWSKRSVVGWPT
KSKRSVVGWPTVRER
SVVGWPTVRERMRRA
WPTVRERMRRAEPAA
RERMRRAEPAADGVG
RRAEPAADGVGAVSR
PAADGVGAVSRDLEK
GVGAVSRDLEKHGAI
(10) LEKHGAITSSNTAAN
GAITSSNTAANNADC
SSNTAANNADCAWLE
AANNADCAWLEAQEE
ADCAWLEAQEEEEVG
WLEAQEEEEVGFPVR
QEEEEVGFPVRPQVP
EVGFPVRPQVPLRPM
PVRPQVPLRPMTYKA
QVPLRPMTYKAAVDL
(20) RPMTYKAAVDLSHFL

YKAAVDLSHFLKEKG
VDLSHFLKEKGGLEG
HFLKEKGGLEGLIYS
EKGGLEGLIYSQKRQ
LEGLIYSQKRQDILD
IYSQKRQDILDLWVY
KRQDILDLWVHTQG
ILDLWVHTQGYFPD
WVHTQGYFPDQNY
(30) TQGYFPDQNYTPGQ
FPDQNYTPGQIRY
QNYTPGQIRYPLTF
PGQIRYPLTFGWCF
IRYPLTFGWCFKLVP
LTFGWCFKLVPVEPE
WCFKLVPVEPEKVEE
LVPVEPEKVEEANEG
EPEKVEEANEGENNS
VEEANEGENNSLLHP
(40) NEGENNSLLHPMSLH

NNSLLHPMSLHGQDDPEREVLVWKFDSRLAPWHGARLHPEYYKDC

(41) NNSLLHPMSLHGQDD
LHPMSLHGQDDPERE
SLHGQDDPEREVLVW
HGQDDPEREVLVWKFDS
EREVLVWKFDSRLAP
LVWKFDSRLAPWHRA
FDSRLAPWHGARLH
LAPWHGARLHPEYY
(49) WHGARLHPEYYKDC

FIGURE 14

FFREDLAFQGKAREFSSEQTRANSPTRRELQVWGRDNNSLSEAGADRQGTVSFSFPQITLMQRPLVTIKIGGQLKEALLDTGADDTVLEEMNLPGRWKPKNIGGIGGFIKVRQYDQILIEICGHKAIGTVLVGPTPVNIIGRNLLTQIGCTLNFPISPIETVPVKLKPGM

(1) FFREDLAFQGKAREFSSE

DLAFQGKAREFSSE

FQGKAREFSSEQTRA

AREFSSEQTRANSPT

SSEQTRANSPTRREL

TRANSPTRRELQVRG

SPTRRELQVWGRDNN

RELQVWGRDNNSLSE

VWGRDNNSLSEAGAD

(10) DNNSLSEAGADRQGT

LSEAGADRQGTVSFS

GADRQGTVSFSFPQI

QGTVSFSFPQITLMQ

SFSFPQITLMQRPLV

FQITLMQRPLVTIKI

LMQRPLVTIKIGGQL

PLVTIKIGGQLKEAL

IKIGGQLKEALLDTG

GQLKEALLDTGADDT

(20) EALLDTGADDTVLEE

DDTVLEEMNLP

(21) DTGADDTVLEEMNLP

LEEMNLPGRWK

NLPGRWKPKNI

RWKPKNIGGIGG

KNIGGIGGFIKVRQY

GIGGFIKVRQYDQIL

FIKVRQYDQILIEIC

RQYDQILIEICGHKA

(30) QILIEICGHKAIGTV

EICGHKAIGTVLVGP

HKAIGTVLVGPTPVN

GTVLVGPTPVNIIGR

VGPTPVNIIGRNLLT

PVNIIGRNLLTQIGC

IGRNLLTQIGCTLNF

LLTQIGCTLNFPISP

IGCTLNFPISPIETV

LNFPISPIETVPVKL

(40) ISPIETVPVKLKPGM

ETVPVKLKPGDGPKVKQWPLTEEKIKALVEICTEMEKEGKISKIGPENPYNTPVFAIKKKDSTKWRKLVDFRELNKRTQDFWEVQLGIPHPAGLKKKKSVTVLDVGDAYFSVPLDKDFRKYTAFTIPSINNETPGIRYQYNVLPQGWKGSPAIFQSSMTKILEPFRKQNP

(41) ETVPVKLKPGDGPK

VKLKPGDGPKVKQW

PGDGPKVKQWPLTE

GPKVKQWPLTEEKI

KQWPLTEEKIKALV

LTEEKIKALVEICTE

KIKALVEICTEMEK

LVEICTEMEKEGKIS

CTEMEKEGKISKIGP

(50) EKEGKISKIGPENPY

KISKIGPENPYNTPV

IGPENPYNTPVFAIK

NPYNTPVFAIKKKDS

TPVFAIKKKDSTKWR

AIKKKDSTKWRKLVD

KDSTKWRKLVDFREL

KWRKLVDFRELNKRT

LVDFRELNKRTQDFW

RELNKRTQDFWEVQL

(60) KRTQDFWEVQLGIPH

(61) DFWEVQLGIPHPAGL

VQLGIPHPAGLKKKK

IPHPAGLKKKKSVTV

AGLKKKKSVTVLDVG

KKKSVTVLDVGDAYF

VTVLDVGDAYFSVPL

DVGDAYFSVPLDKDF

AYFSVPLDKDFRKYT

VPLDKDFRKYTAFTI

(70) KDFRKYTAFTIPSIN

KYTAFTIPSINNETP

FTIPSINNETPGIRY

SINNETPGIRYQYNV

ETPGIRYQYNVLPQG

IRYQYNVLPQGWKGS

YNVLPQGWKGSPAIP

PQGWKGSPAIFQSSM

KGSPAIFQSSMTKIL

AIFQSSMTKILEPFR

(80) SSMTKILEPFRKQNP

FIGURE 15

(161) IEGSETYYTDGSCNKQSKEGKAGYITDKGKDKVKVLSQTNQQAELEAFLMALTDSGPKANIIVDSQYVNGIITGCPTESESSRLVNQIIHEMIKKSEIYVAWVPAHKGIGGNQEIDHLVSQGIRQVLFLEKIEPAQSEHDKYHSNVKELVFKFGLPRIVARQIVDTCDKCH

(181) ESRLVNQIIHEMIKK

(170) EQTTNQQAELEAFLM

(180) FTESESSRLVNQIIEB

(190) HLVSGGIRQVLFLEK

(200) RIVARQIVDTCDKCH

(41) ETVPVKLKPGMDGPKVKQWPLTEEKIKALVEICTENEKEGKISKIGPENPYNTPVFAIKKKDSTKWRKLVDFRELNKRTQDFWEVQLGIPHPAGLKKKKSVTVLDVGDAYFSVPLDKDFRKYTAFTIPSINNETPGIRYQYNVLPQGWKGSPAIFQSSMTKILEPFRKQNP

(61) DFWEVQLGIPHPAGL

(70) KDFRKYTAFTIPSIN

(60) KKTQDFWEVQLGIPH

(80) SSMTKILEPFRKQNP

(501) EKEGKISKIGPENPY

FIGURE 15 contd

FIGURE 15 contd

(161) LQDSGLEVNIVTDSQYALGIIQAQPDKSESSELVSQIIEQLIKKEKVLAWVPAHKGIGGNEQVDKLVSACIRKVLFLGGIDKAQEEHEKYHSNMRAMASDFNLPPVVAKSIVASCDKCQLKGEAMHGQVDCSPGIWQLDCTHLEGKIILVAVVWASGYIEAEVIPAETGQE

LQDSGLEVNIVTDSQ
GLEVNIVTDSQYALG
NIVTDSQYALGIIQA
DSQYALGIIQAQPDK
ALGIIQAQPDKSESE
IQAQPDKSESSELVSQ
PDKSESSELVSQIIEQ
ESELVSQIIEQLIKK
VSQIIEQLIKKEKVY
(170) IEQLIKKEKVLAWV
IKKEKVLAWVPAHK
KVLAWVPAHKGIGG
AWVPAHKGIGGNEQV
AHKGIGGNEQVDKLV
IGGNEQVDKLVSACI
EQVDKLVSACIRKVL
KLVSACIRKVLFLLG
AGIRKVLFLLGGIDKA
KVLFLGGIDKAQEEH
(180) LGGIDKAQEEHEKYH

EEHEKYHSNMRAMS
KYHSNMRAMASDFNL
NMRAMASDFNLPPVV
MASDFNLPPVVAKSI
FNLPPVVAKEIVASC
PPVVAKEIVASCDKCQ
KEIVASCDKCQLKGE
ASCDKCQLKGEAMHG
(190) KCQLKGEAMHGQVDC
KGEAMHGQVDCSPGI
MHGQVDCSPGIWQLD
VDCSPGIWQLDCTHL
PGIWQLDCTHLEGKI
QLDCTHLEGKIILVA
THLEGKIILVAVVVA
GKIILVAVVVASGYI
LVAVVVASGYIEAEV
HVASGYIEAEV
(200) GYIEAEVIPAETGQE

(201) AEVIPAETGQETAYFLLKLAGRWPVKTIHDNGSNFTSTVKAACWNAGIKQEPGIPYNPQSQGVVESNNKELKKIIGQVEDQAEHLKTAVQMAVPIHNFKRKGGIGGYSAGERIVDIIATDIQTKELQKQITKIQNFRVYRDSRDPLWKGPAKLLNKGEGAVVIQDNSDI

AEVIPAETGQETAYF
PAETGQETAYFLLKL
GQETAYFLLKLAGRW
AYFLLKLAGRWPVKT
LKLAGRWPVKTIHTD
GRWPVKTIHTDNGSN
VKTIHTDNGSNFTST
IHTDNGSNFTSTVKA
GSNFTSTVKAACW
(210) TSTVKAACWNAGIK
VKAACWNAGIKQEPG
CWNAGIKQEPGIPYN
GIKQEPGIPYNPQSQ
EFGIPYNPQSQGVVE
PYNPQSQGVVESNNK
QSQGVVESNNKELKK
VVESNNKELKKIIGQ
NNKELKKIIGQVEDQ
LKKIIGQVEDQAEH
(220) IGQVEDQAEHLKTAV

EHLKTAVQMAVPIHN
EHLKTAVQMAVPV
TAVQMAVPIHNFK
MAVPIHNFKRKGGIG
IHNFKRKGGIGGYSA
KRKGGIGGYSAGERI
GIGGYSAGERIVDII
YSAGERIVDIIATDI
ERIVDIIATDIQTKE
(230) DIIATDIQTKELQKQ
TDIQTKELQKQITKI
TKELQKQITKIQNFR
QKQITKIQNFRVYRD
TKIQNFRVYRDSRDP
NFRVYRDSRDPLWKG
YRDSRDPLWKGPAKL
RDPLWKGPAKLLNKG
WKGPAKLLNKGEGAV
AKLLNKGEGAVVIQD
NKGEGAVVIQDNSDI
(240) WKGEGAVVIQDNSDI

FIGURE 15 contd

GAVVIQDNSDIKVPRRKAKIIRDYGKQMAGDDCVASRQDBD

[241] GAVVIQDNSDIKVP

IQDNSDIKVPRRKA

SDIKVPRRKAKIIR

VVPRRKAKIIRDYGK

RKAKIIRDYGKQMAG

IIRDYGKQMAGDDCV

YGKQMAGDDCVASRQ

(248) NRGDDCVASRQDBD

FIGURE 15 contd

FIGURE 16

(1) MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKGLGISYGRKKRRQRRRAPQDSQTHQVSLSKQPASQPRGDPTGPKESKKKVGRETETDPVDQ
(11) MEPVDPRLEPWKHPG
DPRLEPWKHPGSQPK
EPWKHPGSQPKTACT
HPGSQPKTACTNCYC
QPKTACTNCYCKKCC
ACTNCYCKKCCFHCQ
CYCKKCCFHCQVCFI
KCCFHCQVCFITKGL
HCQVCFITKGLGISY
(10) CFITKGLGISYGRKK
KGLGISYGRKKRRQR
ISYGRKKRRQRRRAP
RKKRRQRRRAPQDSQ
RQRRRAPQDSQTHQV
RAPQDSQTHQVSLSK
DSQTHQVSLSKQPAS
HQVSLSKQPASQPRG
LSKQPASQPRGDPTG
(20) PASQPRGDPTGPKES

(21) PRGDPTGPKESKKKV
PTGPKESKKKVGRET
KESKKKVGRETETDP
(24) KKVGRETETDPVDQ

FIGURE 17

MEMRHQVNIVHQVDRMRIRTWKSLVKHHWYISRKAKGHFYRHHYESTHPIRLSSEVHIPLGDARLJVTTYWGLHTGERDKWHLGQGVSIEWRKKRYSTQVDPDLADQLIHLYYPDCFSESAIRMAILGHIVSPRCEYQAGHMKVGSLQYLALAALITPKKIKPPLPSVTKLTE

(1) MEMRHQVNIVHQVDR
MQVNIVHQVDR
IVHQVDRMRIRTWKS
VDRMRIRTWKSLVKH
RIRTWKSLVKHHWYI
WKSLVKHHWYISRKA
VKHHWYISRKAKGFP
WYISRKAKGFYRHH
RKAKGHFYRHHYEST
(10) GHFYRHHYESTHPRI
RHHYESTHPRLSSEV
ESTHPRLSSEVHIPL
PRLSSEVHIPLGDAR
SEVHIPLGDARLJVT
IPLGDARLVITTYWG
DARLVITTYKGLHTG
VITTYWGLHTGERDK
YWGLHTGERDKWHLGQ
HTGERDKWHLGQGVSI
(20) RDKWHLGQGVSIEWRK

VSIEWRKKRYSTQVD
WRKKRYSTQVDPDLA
RYSTQVDPDLADQLI
QVDPDLADQLIHLYY
DLADQLIHLYYPDCF
QLIHLYYFDCFSESA
LYYFDCFSESAIRNA
DCFSESAIRNAILGH
(30) ESAIRNAILGHIVSP
RNAILGHIVSPRCEY
LGHIVSPRCEYQAGH
VSPRCEYQAGHMKVG
CEYQAGHMKVGSLQY
AGHMKVGSLQYLALA
MKVGSLQYLALAALIT
LQYLALAALITPKKI
ALAALITPKKIKPPL
LITPKKIKPPLPSVT
(40) KKIKPPLPSVTKLTE

(21) LGQGVSIEWRKKRYS

PPLPSVTKLTEDRMNKPQKTKGHRGSHTYNGH
(41) PPLPSVTKLTEDRMN
SVTKLTEDRMNKPQK
LTEDRMNKPQKTKGH
RMNKPQKTKGHRGSH
PQKTKGHRGSHTYNG
(46) KGHRGSHTYNGH

FIGURE 18

```
(1) MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHGLGQHIYETYGDTWAGVEAIIRILQQLLFIHFRIGCQHSRIGITRQRRARRNGASRS
    MEQAPEDQGPQREPY
       PEDQGPQREPYNEWT
          GPQREPYNEWTLELL
             EPYNEWTLELLEELK
                EWTLELLEELKSEAV
                   ELLEELKSEAVRHFP
                      EAVRHFPRIWLHGLG
                         HFPRIWLHGLGQHIY
(10)                        IWLHGLGQHIYETYG
                               GLGQHIYETYGDTWA
                                  HIYETYGDTWAGVEA
                                     TYGDTWAGVEAIIRI
                                        TWAGVEAIIRILQQL
                                           VEAIIRILQQLLFIH
                                              IIRILQQLLFIHFRIG
                                                 QQLLFIHFRIGCQHS
                                                    FIHFRIGCQHSRIGI
                                                       RIGCQHSRIGITRQR
(10)                                                      QHSRIGITRQRRARN
(21) IGITRQRRARRNGASR
(22) RQRRARRNGASRS
```

FIGURE 19

```
     MQSLQILAIVALVVAAIIAIVVHSIVFIEYRKILRQRKIDRLIDRIRERAEDSGNESEGDQEELSALVEMGHHAPWDVDDL
(1)  MQSLQILAIVALVVA
     QILAIVALVVAAIIA
     IVALVVAAIIAIVVH
     VVAAIIAIVVHSIVP
     IIAIVVHSIVFIEYR
     VVHSIVFIEYRKILR
     IVFIEYRKILRQRKI
     EYRKILRQRKIDRLI
     ILRQRKIDRLIDRIR
(10) RKIDRLIDRIRERAE
     RLIDRIRERAEDSGN
     RIRERAEDSGNESEG
     RAEDSGNESEGDQEE
     SGNESEGDQEELSAL
     SEGDQEELSALVEMG
     QEELSALVEMGHHAP
     SALVEMGHHAPWDVD
(18) EMGHHAPWDVDDL
```

FIGURE 20

```
(1) NSTNPKPQRKTKRNTRRRPQDVKFPGGGQIVGGVTLLPRRGPRLGVRATRKTSBERSQFRGRRQPIPKQARRPEGRKTWAQPGYFWPLYGHESCGHAGHLLSPRGSRSRSWGPTDPRRRSRSRGLGKVIDTLTCGFADLWGYIYIPLVGAPLCGHARAL
     NSTNPKPQRKTKRNTRRR
     QRKTKRNTRRRPQDVKFP
                       TRRRRPQDVKFPGGGQIVG       QPGYFWPLYGHESCGHAG
(11) PEGRKTWAQPGYFWPLYGH
                       VKFPGGGQIVGGVTLLPR        LYGHESCGHAGHLLSPRG
       QIVGGVTLLPRRGPRLGV          GHAGHLLSPRGSRPSWGP
         LLPRRGPRLGVRATRKTS           SPRGSRSRSWGPTDPRRRS
           RLGVRATRKTSBERSQPRG            SWGPTDPRRRSRSRGLGKVI
             RKTSBERSQPRGRRQPIPK            RRRSRSRGLGKVIDTLTCGP
               QPRGRRQPIPKQARRPEGR              GKVIDTLTCGFADLWGYI
(10) PIPKQARRPEGRKTWAQPGY                       TCGFADLWGYIPLVGAPL
                                           (20) WGYIPLVGAPLCGHARAL

(21) GAPLCGHARALAHGVRVLRDGVNYATGHLPGCSFSIFLLALLSCLTVPASAYQVRNSSGLYHVTNDCFNSSIVEAADAILHTPGCVPCVREGNASRCHVAVTPTVATRNASRCHVAVTPTVATRDGKLFTTQLRRHIDLLVGSATLCSALYVGDLCGSV
     GAPLCGHARALAHGVRVL
       ARALAHGVRVLRDGVNYA
         VRVLRDGVNYATGHLPGC           DAILHTPGCVPCVREGNA
         VNYATGHLPGCSFSIFLL        (31) TNDCFNSSIVEAADAIL          GCVPCVREGNASRCHVAV
               LPGCSFSIFLLALLS            SIVEAADAILHTPGCVP            SRCHVAVTPTVATR
                 SFSIFLLALLSCLT                                         WVAVTPTVATRDGKLFTT
                   IFLLALLSCLTVPASAYQ                                   VATRDGKLFTTQLRRHID
                     SCLTVPASAYQVRNSSGL                                   LFTTQLRRHIDLLVGSAT
                       SAYQVRNSSGLYHVTNDC                                   RHIDLLVGSATLCSALYV
(30) SSGLYHVTNDCFNSSIVY                                             (40) GSATLCSALYVGDLCGSV

(41) ALYVGDLCGSVFLVGQLFTTSPRRHNTTQDCNCSIYPGHITCHRNANRRQNNHEPTALLVVAQLLRIRQAIMDMIAGNHWDVLAGIAYPSMVGSNAKVLVVLLLFAGVDAETHVTGGSAGRTYAGLVGLLTPGAKQNIQLINTRGSWHINS
     ALYVGDLCGSVFLVGQLF
       CGSVFLVGQLFTTSPRRH         AHWDVLAGIAYPSMVGSN
         GQLFTTSPRRHNTTQDCN      (51) IMDMIAGNHWDVLAGIAY         VGSNAKVLVVLLLFAGVD
           PRRHNTTQDCNCSIYPGH            GIAYPSMVGSNAKVLVVL            LVVLLLFAGVDAETHVTG
             QDCNCSIYPGHITCHRNA                                         AGVDAETHVTGGSAGRTY
               YPGHITCHRNANRRQNNH                                       HVTGGSAGRTYAGLVGLL
                 HRNANRRQNNHSPTAILV                                       GRTYAGLVGLLTPGAKQN
                   NNHSPTAILVVAQLLRI                                      VGLLTPGAKQNIQLINTR
(50) LLRIRQAIMDMIAGNHWD                                            (60) AKQNIQLINTRGSWHINS

(61) IGTNGSWHINSTALNCHESLATGNLAGLFYQHKPVSSGCPERLASCRRLTDFAQGCPISYANGSGLDERPYCHHYPRPCGIVPAKSVCGPVYCFTSPVVVGTTDRSGAPTYSNGANDTDVFVLANTRPPLGNWPGCTVKNSTGFTKVC
     IGTNGSWHINSTALNCHE
       HINSTALNCHESLATGNL        (71) PYCHHYPRPCGIVPAKS
         NCHESLATGNLAGLFYQH             PRPCGIVPAKSVCGPVYC           PAKSVCGPVYCFTSPVV
           TGNLAGLFYQHKPVSSGC            FVYCFTSPVVVGTTDRS            SPVVVGTTDRSGAPTYSN
             FYQHKPVSSGCPERLASC                                       TDRSGAPTYSNGANDTDV
               SSGCPERLASCRRLTDFA                                       TYSNGANDTDVFVLANTR
                 LASCRRLTDFAQGCPIS                                      DTDVFVLANTRPPLGNWP
                   TDFAQGCPISYANGSGL                                    NNTRPPLGNWPGCTVKNS
(70) GSGLDERPYCHHYPRPC                                             (80) GNWPGCTVKNSTGFTKVC
```

FIGURE 21

MHNSTGFTKVCGAPCVIGGVGNNTLLCPTDCFRGHPEATTSRCCGSGPMITPRCMVDYPYRLMHYPCTINYTIFKVRMVVGGVEHRLEAACMWTRGSRCDLEDRDRSRLSPLLLSTTQHQVLPCSFTTLPALSTGLIHLHQFIVDVQYLVG
(81) MHNSTGFTKVCGAPCVI

TKVCGAPCVIGGVGNNT
PCVIGGVGNNTLLCPTDC
GGNNTLLCPTDCFRGHPEA
(91) YTIFKVRMVVGGVEHRLE
MVVGGVEHRLEAACNWTR

PTDCFRGHPEATYSRCGS
HRLEAACMWTRGSRCDLE
HPEATYSRCCGSGPMITPR
CDLEDRDRSRLSPLALST
RCCGSGPMITPRCMVDYPY
RSRLSPLLLSTTQHQVLP
ITPRCMVDYPYRLMHYPC
LLSTTQHQVLPCSFTTLP
DYPYRLMHYPCTINYTIF
QVLPCSFTTLPALSTGLI
(90) HYPCTINYTIFKVRMVVG
TTLPALSTGLIHLHQNIV
(100) TGLIHLHQNIVDVQYLVG

QNIVDVQYLVGGVGSSIASNAIKWETVVLLFLLLADARVCSCLMMHLLISQSEAALENLVILNAASLAGTHGLVSFLVFFCFAMVLKGRHVPGAVYAFYGNWPFLLLLLALPQRAYALDEVAASCGGVVLVGLMALTLSPYYKRYISNCMH
(101) QNIVDVQYLVGGVGSSIAS
(111) GLVSFLVFFCFAMVLKGR

YLVGGVGSSIASNAIKWEY
FFCFAMVLKGRHVPGAVY
SIASNAIKWETVVLLFLL
LKGRHVPGAVYAFYGNWP
KWETVVLLFLLLADARVC
GAVYAFYGNWPFLLLLLA
LFLLLADARVCSCLMMHL
GNWPFLLLLLALPQRAYA
ARVCSCLMMHLLISQSEA
LLLALPQRAYALDEVAA
MMHLLISQSEAALENLVI
RAYALDEVAASCGGVVL
QSEAALENLVILNAASLA
EVAASCGGVVLVGLMALT
NLVILNAASLAGTHGLVS
GVVLVGLMALTLSPYYK
(110) ASLAGTHGLVSFLVFFCF
(120) MALTLSPYYKRYISNCMH

TYYKRYISNCMHRLQYFIRVEAQLHVNVPPLAVRGGRDAVILLMCVVHPTLVFDITKLLLAIFGPLNILQASLLKVPYFVRVQGLLRICALARKIAGGHRVQNAIIKLGALIGTVYVNHLTPLRDWAHNGLRDLAVAVBPVVFSRNETKLI
(121) TYYKRYISNCMHRLQYFLI
(131) ASLLKVPYFVRVQGLLRI

NCMHRLQYFIRVEAQLH
YFVRVQGLLRICALARKI
YFIRVEAQLHVNVPPLN
LLRICALARKIAGGHRVQ
AQLHVNVPPLAVRGGRDA
ARKIAGGHRVQNAIIKLG
PLAVRGGRDAVILLMCV
HRVQNAIIKLGALIGTV
GRDAVILLMCVVHPTLVF
IKLGALIGTVYVNHLTPL
LMCVVHPTLVFDITKLL
GTVYVNHLTPLRDWAHNG
TLVFDITKLLLAIFGPLN
LTPLRDWAHNGLRDLAVA
KLLLAIFGPLNILQASLL
AHNGLRDLAVAVEPVVFS
(130) GPLNILQASLLKVPYFVR
(140) LAVAVEPVVFSRNETKLI

VVFSRNETKLITWGADTRACGDIINGLPVSARRGQBILLGPADGMVSKGHRLLAPITAYAQQTRGLLGCIITSLHGRDRNQVEGEVQIVSTATQYFLATCINGVCNTVYHGAGTRTIASPKGPVIQMYTNVDQDLVGHPAPQGS
(141) VVFSRNETKLITWGADTA
(151) IISLHGRDRNQVEGEVQI

TKLITWGADTRACGDIIN
DRNQVEGEVQIVSTATQY
ADTRACGDIINGLPVSAR
EVQIVSTATQYFLAT
DIINGLPVSARRGQBILL
VSTATQYFLATCIN
VSARRGQBILLGPADGMV
ATQYFLATCINGVCNTVY
BILLGPADGMVSKGHRLL
TCINGVCNTVYHGAGTRT
DGMVSKGHRLLAPITAYA
WTVYHGAGTRTIASPKGP
HRLLAPITAYAQQTRGLL
GTRTIASPKGPVIQMYTN
TAYAQQTRGLLGCIITSL
PKGPVIQMYTNVDQDLVG
(150) RGLLGCIITSLHGRDRNQ
(160) MTNVDQDLVGHPAPQGS

FIGURE 21 contd

[161] DLVGHPAPQGSRSLTPCTCGSSDLYLVTRHADVIPVRRRGDSRGSLLSPRPISYLKGSSGGPLLCPAGHAVGLFRAAVCTRGVAKAVDPIPVEHLETTHRSPVFTDNSSPPAVRQSFQVAHLHAPTGSGKSTKVPAAYAAQGYKVLVLNPS
DLVGHPAPQGSRSLTPCT
PQGSRSLTPCTCGSSDLY
TPCTCGSSDLXLVTRHAD
SDLYLVTRHADVIPVRRR
RHADVIPVRRRGDSRGSL
VRRRGDSRGSLLSPRPIS
RGSLLSPRPISYLKGSSG
RPISYLKGSSGGPLLCPA
GSSGGPLLCPAGHAVGLF
[170] LCPAGHAVGLFRAAVCTR
(171) VGLFRAAVCTRGVAKAVD
VCTRGVAKAVCTKGVRAVD
KAVDFIPVEHLETTHRSP
VENLETTHRSPVFTDNSS
HRSPVFTDNSSPPAVRQS
DNSSPPAVRQSFQVAHLH
VRQSFQVAHLHAPTSGGK
AHLHAPTSGGKSTKVPAA
GSGKSTKVPAAYAAQGYK
(1801) VPAAYAAQGYKVLVLNPS

[181] QGYKVLVLNPSVAATLGFGAYHSKAEGVDPHIRKGVRTITTGSPITYSTYGKFLADGGCSGGAYDIIICDECHSTDATSILGIGTVLDQAETAGARLVVLATATPPGSVTVSHPNIEEVALSTTGEIPFYGKAIPLSVIKGGRHLIFCHSK
OGYKVLVLNPSVAATLGF
LNPSVAATLGFGAYHSKA
TLGFGAYHSKAGVDPHI
MSKAGVDPHIRKGVRTI
DPHIRKGVRTITTGSPIT
VRTITTGSPITYSTYGKF
SPITYSTYGKFLADGGCS
YGKFLADGGCSGGAYDII
GGCSGGAYDIIICDECHS
(190) YDIIICDECHSTDATSIL
(191) ECHSTDATSILGIGTVLD
TSILGIGTVLDQAETAGA
TVLDQAETAGARLVVLAT
TAGARLVVLATATPPGSV
VLATATPPGSVTVSHPNI
PGSVTVSHPNIEEVALST
HPNIEEVALSTTGEIPFY
ALSTTGEIPFYGKAIPLE
IPFYGKAIPLEVIKGGRH
(200) IPLEVIKGGRHLIFCHSK

[201] GGRHLIFCHSKKKCDELAAKLVALGINAVAYYRGLDVSSVIPTSGDVVVVSTDALMTGFTGDFDSVIDCNTCVTQVDFSLDPFFTIETTTLPQDAVSRTQRGRTGRGKRGIYRPVAPGERPSGKFDSSVLCECYDAGCAWYELTPAE
GGRHLIFCHSKKKCDELA
CHSKKKCDELAAKLVALG
DELAAKLVALGINAVAYY
VALGINAVAYYRGLDVSV
VAYYRGLDVSVIPTSGDV
DVSVIPTSGDVVVVSTDA
SGDVVVVSTDALMTGFTG
STDALMTGFTGDFDSVID
GFTGDFDSVIDCNTCVTQ
(210) SVIDCNTCVTQVDFSLD
(211) CVTQVDFSLDPFFTIET
FSLDPFFTIETTTLPQDA
TIETTTLPQDAVSRTQR
PQDAVSRTQRGRTGRGK
TQRGRTGRGKRGIYRPV
GRGKRGIYRPVAPGERPS
YRPVAPGERPSGKFDSSV
ERPSGKFDSSVLCECYDA
DSSVLCECYDAGCAWYEL
(220) CYDAGCAWYELTPAE

[221] GCAWYELTPAETTVRLRAYMNTPGLPVCQDHLEFEGVFTGLTHIDAHFLSQTKQSGENRPPYLVAYQATVCARAQAPPPSWDQMKCLIRLKPTLHGPTPLLYRLGAVQNEVLTHPITKYIMTCMSADLEVTSTVVLVGGVLAAL
GCAWYELTPAETTV
WYELTPAETTVRLRAY
STTVRLRAYRNTPGLPVC
AYMNTPGLPVCQDHLEFN
LPVCQDHLEFNHGVFTGL
LEFNHGVFTGLTHIDAHF
FTGLTHIDAHFLSQTKQS
DAHFLSQTKQSGENRPPYL
TKQSGENRPPYLVAYQATV
(230) FPYLVAYQATVCARAQAP
(231) QATVCARAQAPPPSWDQM
AQAPPPSWDQMKCLIRL
HDQMKCLIRLKPTLHGP
LIRLKPTLHGPTPLLYRL
LHGPTPLLYRLGAVQNEV
LYRLGAVQNEVLTHPIT
QNEVLTHPITKYIMTCM
HPITKYIMTCMSADLEV
MTCMSADLEVTST
(240) TSTVVLVGGVLAAL

FIGURE 21 contd

(241) NVLVGGVLAALAAYCLSTGCVVIVGRIVLSGKPAIIPDREVLYQEFDEMEECSQHLPYIEQGMGLAEPFQKNALGLLQTASRQAEVITPAVQTNWQKLEVFRAKHWNFISGIQYLAGLSTLPGNPAIASLMAFTAA
NVLVGGVLAALAAYCLST
LAALAAYCLSTGCVV
AAYCLSTGCVVIVG
CLSTGCVVIVGRIVL
GCVVIVGRIVLSGK
VIVGRIVLSGKPAIIPDR
LSGKPAIIPDREVLYQEF
IPDREVLYQEFDEMEECS
YQEFDEMEECSQHLPYIE
(250) EECSQHLPYIEQGMGLAE
(251) PYIEQGMGLAEPFQKNAL
MGLAEPFQKNALGLLQTAS
QKNALGLLQTASRQAEVIT
QTASRQAEVITPAVQTNW
EVITPAVQTNWQKLEVFW
QTNWQKLEVFWAKHWNP
EVFWAKHWNPISGIQYL
WNPISGIQYLAGLSTLP
IQYLAGLSTLPGNPAIAS
(260) STLPGNPAIASLMAFTAA

(261) AIASLMAFTAAVTSPLITGQTLLFNILGGNVAQLAAPGAATAPVGAGLAGAAIGSVGLGKVLVDILAGYGAGVAGALVAFKIWSGEVPSTEDLVNLLPAILSPGALVVGVVCAAILRRHVGPGEGAVQWNNRLIAFASRGNHVSPTHYVP
AIASLMAFTAAVTSPLIT
FTAAVTSPLITGQTLLFN
PLITGQTLLFNILGGNVA
LLFNILGGNVAQLAAPG
GNVAQLAAPGAATAPVG
AAPGAATAPVGAGLAGAA
AFVGAGLAGAAIGSVGLG
AGAAIGSVGLGKVLVDIL
VGLGKVLVDILAGYGAGV
(270) VDILAGYGAGVAGALVAF
(271) GAGVAGALVAFKIWSGEV
LVAFKIWSGEVPSTEDLV
SGEVPSTEDLVNLLPAIL
EDLVNLLPAILSPGALVV
PAILSPGALVVGVVCAAI
ALVVGVVCAAILRRHVGP
CAAILRRHVGPGEGAVQW
HVGPGEGAVQWNNRLIAP
AVQWNNRLIAFASRGNHV
(280) LIAFASRGNHVSPTHYVP

(281) GNHVSPTHYVPESDAAAVTAILSSLIVTQLLRRLHQWISSECTTPCSGSWLRDINDHICEVLSDFKTVLKAXLMPQLPGIPFVSCQRGYRGVVRGDGIHETRCHCGAAITGHVANGTARIVGPRTCRMNWSGTFPINAYTTGPCTPLPAP
GNHVSPTHYVPESDAAAV
HVYPESDAAAVTAILSS
AAAVTAILSSLIVTQLL
ILSSLIVTQLLRRLHQI
TQLLRRLHQWISSECTTP
RQWISSECTTPCSGSWLR
CTTPCSGSWLRDINDHIC
SWLRDINDHICEVLSDFK
DHICEVLSDFKTVLKAXL
(290) SDFKTVLKAXLMPQLPGI
(291) KAXLMPQLPGIPFVSCQR
LPGIPFVSCQRGYRGVVR
SCQRGYRGVVRGDGIHGT
GVVRGDGIHGTRCHCGAA
IGHTRCHCGAAITGHVHN
CGAAITGHVHNGTARIVG
HVHNGTARIVGPRTCRHN
RIVGPRTCRHNWSGTFPI
CRHNWSGTFPINAYTTGP
(300) TFPINAYTTGPCTPLPAP

(301) TTGPCTPLPAPNYKFALRVSABEYVEIRVGDFHYVSGWTTDNLKCPCQIPSPBFFTELDGVRLHRFAPCKPLLREEVSFRVGLHBYPVGSQLPCBPZPDVAVLTEMLTDPSHITABAAGRRLARGSPESWASSSASASQLSAPSLKAYCT
TTGPCTPLPAPNYKFALH
LPAPNYKFALRVSABEY
FALRVSABEYVEIRVG
AABEYVEIRVBIERVG
RRVGDFHYVSGWTTDNLK
YVSGWTTDNLKCPCQIPS
DNLKCPCQIPSPBFFTEL
QIPSPBFFTELDGVRLHR
FTELDGVRLHRFAPCKP
(310) RLHRFAPCKPLLREEVS
(311) PCKPLLREEVSFRVGLHE
EEVSFRVGLHEYPVGSQL
GLHEYPVGSQLPCBPZPD
GSQLPCBPZPDVAVLTEM
PZPDVAVLTEMLTDPSHI
LTEMLTDPSHITABAAGR
PSHITABAAGRRLARGSP
AAGRRLARGSPESWASS
RGSPESWASSSASQLSAP
(320) ASSSASQLSAPSLKAYCT

FIGURE 21 contd

(321) LSAPSLKATCTANHDSPDAELIEANLLRRQEQGNITRVESENKVVILDSFDPLVABEDEREVSVPAELLRKSRRFARALPVNARPDYNPPLLVETWKKPDYBPPVVHGCPLPPRSPVPPPRKKKRTVVLTESTLSTALAELATKSFGSSS
LSAPSLKATCTANHDSPD
         AICTANHDSPDAELIEAN
                 DSPDAELIEANLLMRQEK
                        IEANLLMRQEKGGNITRV
                              RQEKGGNITRVESENKVV
                                    IIRVESENKVVILDSFDP
                                         NKVVILDSFDPLVABEDE
                                              SFDPLVABEDEREVSVPA
                                                   EEDEREVSVPABILRKSR
                                              (310) SVPABILRKSRRFARALP
                                                                      RKSRRFARALPVNARPDY
                                                              (331) RKSRRFARALPVNARPDY
                                                                  RALPVNARPDYNPPLVET
                                                                      RPDYNPPLVETWKKPDYB
                                                                          LVETWKKPDYBPPVVHGC
                                                                               PDYBPPVVHGCPLPPPRS
                                                                                   VHGCPLPPPRSPVPPPR
                                                                                       PPRSPVPPPRKKKRTVL
                                                                                   (340) TLSTALAELATKSFGSSS
                                                                                            TVVLTESTLSTALAELAT
                                                                                        PPPRKKKRTVVLTESTLST

(341) ELATKSFGSSSTSGITGDNTTYTSSEPAPSGCPPOSDVTSSSMPPLEGBPKDPDLSDGSMPGDSTVSSGADTEDVVCCSNSYSMTGALVTPCAAEEQKLPINALSNSLLRHENIVYSTTSRSACQR
      ELATKSFGSSSTSGITGD
         GSSSTSGITGDNTTTSSE
              ITGDNTTTSSEPAPSGCP
                   TTTSSEPAPSGCPPSDVES
                        SGCPPSDVESYSSM
                             PSDVESYSSMPPL
                                  DVESYSSMPPLEGBPGDP
                                       MPPLEGBPGDPDLSDGSM
                                            PGDPDLSDGSMSTVSSGA
                                       (350) DGSMSTVSSGADTED
                             (351) TVBSGADTEDVVC
                                      SSGADTEDVVCCSNS
                                           DTEDVVCCSNSYSN
                                                DVVCCSNSYSMTGAL
                                                     CSNSYSMTGALVTP
                                                          SYSMTGALVTPCAAEQK
                                                               LVTPCAAEQKLPINALS
                                                                    EEQKLPINALSNSLLRH
                                                                         NALSNSLLRHENIVYSTT
                                                                    (360) LRHENIVYSTTSRSACQR

(361) YSTTSRSACQRQKKVTFDRLQVTLDSHYQDVLKEYVRAAASKVFCWILLSVEEACSLTPPHSAKSKFGYGAKDVRCHARKAVAEINSVNKDLLEDSVTPIDTTIMAKNEVFCVQPEKGGRKPAARLIVFPDLGVRVCERWALYDVVSKLPLAVKG
      YSTTSRSACQRQKKVTFD
         ACQRQKKVTFDRLQVLDS
             VTFDRLQVLDSHYQDVLK
                  VLDSHYQDVLKEYVRAAS
                       VLKEYVRAAASKVFCWILL
                            AAASKVFCWILLSVEEACS
                                 FCWILLSVEEACSLTPPHSA
                                      EACSLTPPHSAKSKFGYG
                                           PHSAKSKFGYGAKDVRCH
                                      (370) FGYGAKDVRCHARKAVAH
                                                    RCHARKAVAEINSVNKD
                                           (371) VRCHARKAVAEINSVNKD
                                                 AVAEINSVNKDLLEDSVT
                                                      VNKDLLEDSVTPIDTTIM
                                                           DSVTPIDTTIMAKNEVFC
                                                                TIMAKNEVFCVQPEKGG
                                                                     EVFCVQPEKGGRKPARLI
                                                                          KGGRKPARLIVFPDLGV
                                                                               ARLIVFPDLGVRVCERWA
                                                                          (380) ERWALYDVVSKLPLAVKG
                                                                                   DLGVRVCERWALYDVVSK

(381) VVSKLPLAVKGSSYGFQYSPGQRVEFLVQAWKSKKTFWGFSYDTRCFDSTVTESDIRTEEAIYQCCDLDPQARVAIKSLTERLYVGGPLTNSRGENCGYRRCRASGVLTTSCGNTLTCYIKARAACRAAGLQDCTMLVCGDDLVVICESSAG
      VVSKLPLAVKGSSYGFQY
         AVKGSSYGFQYSPGQRVE
             GFQYSPGQRVEFLVQAWK
                  QRVEFLVQAWKSKKTFWG
                       QAWKSKKTFWGFSYDTRC
                            TFWGFSYDTRCFDSTVTE
                                 DTRCFDSTVTESDIRTEE
                                      TVTESDIRTEEAIYQCCD
                                           RTEEAIYQCCDLDPQARV
                                      (390) QCCDLDPQARVAIKSLTE
                                                    DPQARVAIKSLTERLYVGG
                                           (391) QARVAIKSLTERLYVGGP
                                                 SLTERLYVGGPLTNSRGE
                                                      VGGPLTNSRGENCGYRRC
                                                           SRGENCGYRRCRASGVLT
                                                                YRRCRASGVLTTSCGNTL
                                                                     GVLTTSCGNTLCYIKAR
                                                                          GNTLTCYIKARAACRAAG
                                                                               IKARAACRAAGLQDCTML
                                                                          (400) CTMLVCGDDLVVICESSAG
                                                                                   RAAGLQDCTMLVCGDDLV

FIGURE 21 contd

(401) DDLVVICESAGVQEDAASLRAPTEANTRYSAPPCDPPQPPEYDLELITSCSENVSVAHDGAGKRVYYLTRDPTTPLARAAWETARHTPVNSHLGNIIMPAFTLMARMILMTHFFSVLIARDQLEQALNCEIYGACYSIEPLDLPP
DDLVVICESAGVQEDAAS
ESAGVQEDAASLRAPTEA
DAASLRAPTEANTRYSAP
FTEANTRYSAPPCDPPQP
YSAPPCDPPQPPEYDLELI
PPQPPEYDLELITSCSENV
LELITSCSENVSVAHDGA
SSENVSVAHDGAGKRVYYL
HDGAGKRVYYLTRDPTTP
(410) VYYLTRDPTTPLARAAWE
PTTPLARAAWETARHTPV
AAWETARHTPVNSHLGNI
HTPVNSHLGNIIMFAPTL
LGNIIMFAPTLMARMILM
APTLMARMILMTHFFSVL
MILMTHFFSVLIARDQLE
FSVLIARDQLEQALNCEI
DQLEQALNCEIYGACYSI
NCEIYGACYSIEPLD
(420) YGACYSIEPLDLPP

(421) CYSIEPLDLPPIIQRLHGLSAFSLHSYSPGEINRVAACLRKLGVPPLRAWRHRARSVRARLLSRGGRAAICGKYLFNWAVRTKLKLTPIAAAGRLDLSGWFTAGVSGGDIYHSVSHARPRWFWFCLLLLAAGVGIYLLPNR
CYSIEPLDLPPIIQRLHG
DLPPIIQRLHGLSAFSLH
RLHGLSAFSLHSYSPGEI
FSLHSYSPGEINRVAACL
PGEINRVAACLRKLGVPP
AACLRKLGVPPLRAWRHR
GVPPLRAWRHRARSVRAR
WRHRARSVRARLLSRGGR
VRARLLSRGGRAAICGKY
(430) RGGRAAICGKYLFNWAVR
CGKYLFNWAVRTKLKLTP
WAVRTKLKLTPIAAAGRL
KLTPIAAAGRLDLSGWFT
AGRLDLSGWFTAGVSGGD
GWFTAGVSGGDIYHSVSH
SGGDIYHSVSHARPRWFW
SVSHARPRWFWFCLLLLA
RWFWFCLLLLAAGVG
FCLLLLAAGVGIYL
(440) LLLAAGVGIYLLPNR

FIGURE 21 contd